

BULLETIN OF THE AGRICULTURAL CHEMICAL SOCIETY OF JAPAN

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Paper Electrophoretic Studies on the Diastatic Enzymes of Molds

Part I. Electrophoretic Technique and the Behaviours of Diastatic Enzymes Observed on Papers.

By Tadahiko ANDO and Yonosuke IKEDA*

The Scientific Research Institute, Bunkyo-ku, Tokyo

Received June 25, 1955

A detailed procedure for the separation of diastatic enzymes of molds was established, which was based on their electrophoretic behaviours on a paper strip. For the detection of enzymes, a bioautographical method was newly devised. Comparing the enzymatic images appeared on the paper strip, the authors classified mold enzyme systems into five types.

In Japan and other Asian countries, various kinds of molds such as *Aspergillus*, *Penicillium*, *Rhizopus*, and *Mucor* are known to be valuable converting agents for the preparation of foods and wines from grains and potatoes. Among numerous reports concerning the mold enzyme system, the following two reports are outstanding owing to their systematic studies. According to Kitahara¹⁾, who investigated the diastatic enzyme systems of molds by using iodine color reaction and saccharifying activity against soluble starch as the assay methods, these molds might be classified into six types in respect to their enzymatic composition, in contrast to Okazaki's view²⁾ who classified the mold enzyme systems into five types by using different strains and another biometric technique.

The experiments which are reported here and the following one were carried out with the same idea as those referred to above, but in a different way to elucidate the actual composition of mold

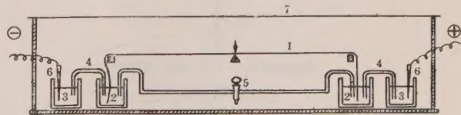
enzyme systems by making them visible on a filter paper.

Methods

Preparation of Enzyme Solution—The following twelve kinds of mold were studied in this experiment,

Aspergillus oryzae (0-8-1), *Asp. sojae* (0-13-8), *Asp. glaucus* (A-1-8), *Asp. usamii* (1-1 R15-0635), *Asp. niger* (A-9-9), *Asp. awamori* (K-2 0913), *Asp. inui* (6911), *Rhizopus tonkinensis* (R-6-4), *Rh. javanicus*, *Rh. péka* (IR-2-4), *Penicillium chrysogenum* (Q-174), *Mucor spinescens* (Mu-3)

The wheat-bran, inoculated with the mold and incubated at 30° for 4 days, was extracted for 15-17 hours with 3 parts of distilled water in the presence of toluene. The enzyme fraction was precipitated from the filtrate by adding absolute alcohol to the final concentration of 75%. The precipitate thus obtained was washed with absolute alcohol and dried in a vacuum-desiccator.



1, filter paper strip; 2, buffer vessel; 3, carbon electrode vessel; 4, 1 M KCl-3% agar bridge; 5, cock; 6, carbon electrode; 7, glass cover.

Fig. 1. Apparatus employed for Electrophoretic Purpose.

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1) K. Kitahara M. Kurushima, *J. Ferm. Tech. Japan* **27** 1, 44, 182, 213, 254 (1949), **28** 106, 388, 422 (1950).

2) H. Okazaki, *J. Agr. Chem. Soc. Japan*, **24**, 88 (1950).

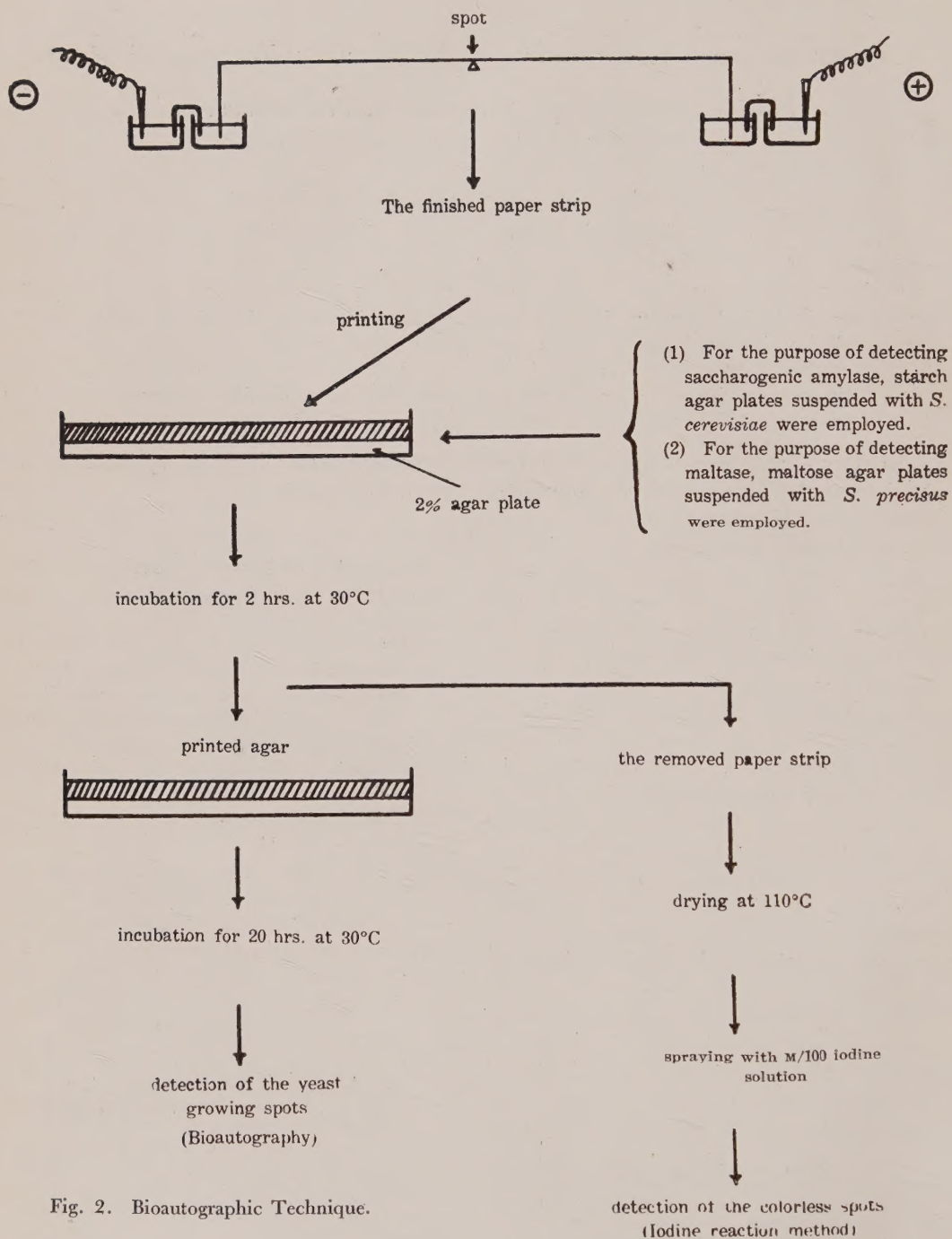


Fig. 2. Bioautographic Technique.

About 0.01ml. aliquot of the enzyme solution was used in the electrophoretic experiment.

Electrophoresis—The apparatus employed in this experiment is shown in Fig. 1. The buffer vessels and the carbon electrode vessels were connected by means of KCl-agar bridges to keep the paper clean from electrode products. By operating the apparatus at the potential gradient of 10 V per cm, satisfactory results were obtained. The strips of paper (Toyo filter paper No. 50), wetted with 0.05 M phosphate buffer and blotted on a dry filter paper to remove any excess moisture, were assembled to the apparatus. When equilibrium reached after thirty minutes, 0.01~0.03 ml. aliquots of the sample were spotted on the starting line of strips, and electromigration was carried out for five hours at room temperature.

Detection of the Enzyme Locus—Two methods were employed for this purpose. The first, rely-

ing on the iodine-starch reaction, served for the detection of dextrinogenic amylase* and the other, relying on bioautographic procedure and being devised by the authors, served for the detection of saccharogenic amylase* and maltase*.

For the detection of saccharogenic amylase, the paper strip was placed on the surface of a thin layer of 2% starch agar plate (medium-D) in which the cells of *Saccharomyces cerevisiae* Rasse II had been previously suspended. After two hours contact, the paper strip was removed. The agar plate thus printed was placed again in an incubator for twenty hours at 30°. (see Fig. 2) During this period, the enzymatic reaction proceeded and the places corresponding to enzyme activities were made visible by the growth of yeast only on saccharified portions of the agar plate. On the whole, it was difficult for us to

* Further definition of these amylases will be given in the following paper.

Table I
Composition of the Media tested for Bioautography.

Constituent	Medium				
	A	B	C	D	E
Asparagine			0.25%		
Peptone				0.5%	1.0%
Beef ext.					1.0%
NH ₄ H ₂ PO ₄		0.2%			
K ₂ HPO ₄	0.5%		0.1 %		
KH ₂ PO ₄		0.2%		0.5%	
MgSO ₄ ·7H ₂ O	0.2%	0.2%	0.3 %	0.2%	
NaCl					0.5%

Soluble starch (as in Table II.) and agar (2%) were supplemented to the individual medium named A~E.

Table II
Selection of Suitable Medium for the Detection of Saccharogenic Amylase.

Medium	Time (hours)	Concentration of Soluble Starch			Remark
		none	1%	2%	
A	20	—	±	±	
B	20	—	+	++	
C	20	—	+	++	
D	20	—	+++	++++	suitable
E	20	—	+++	++++	contamination

The growth rate of *Saccharomyces cerevisiae* Rasse II on these agar plate were recorded, using Taka-diastase as a converting agent.

Table III
Selection of Suitable Test-organism for the Detection of Maltase.

Test-organism	Growth on the agar plate	
	20 hrs.	40 hrs.
<i>Zygosacch.</i> sp. No. 1	+	+++
<i>Zygosacch. saké</i> var K	++	+++
<i>Torulopsis</i> sp. No. 28	+	++
<i>Sacch. presisus</i>	+++	++++

Medium D supplemented with maltose (2%) and agar (2%) was used in this experiment.

detect saccharogenic amylases on a paper strip by the iodine-reaction, but this difficulty was easily overcome by the employment of bioautographic technique, the identification of enzymes being extremely simplified. The compositions of starch agar medium and other related media are shown in Tables I and II.

For the detection of dextrinogenic amylase, the paper strip removed from the agar plate was dried at 110° for five minutes, and then sprayed with 0.01 N iodine solution. The position of dextrinogenic amylase was indicated as a well-defined clear spot depending on the reaction product.

For the detection of maltase, another bioautographic technique was employed. In this case, maltose was added to the medium D instead of soluble starch, and *Sacch. presisus* was employed as test-organism in place of *Sacch. cerevisiae* Rasse II. As shown in Table III, *Sacch. presisus* proved to be the most suitable among these five strains tested, because it showed good growth on the medium only after the added maltose was converted to glucose by maltase.

Results

Electrophoretic Behaviors of Dextrinogenic Amylases—Electromigration was carried out at room temperature for five hours in 0.05 M phosphate buffer (pH 7.6) at the potential of 300 D.C. volts and at the current of 0.3 m. amp/cm. Fig. 3 shows the colored zones of dextrinogenic amylases which appeared on paper strips treated with iodine solution. In contrast to the fact that the spots corresponding to the dextrinogenic amylases of *Asp. oryzae* (+41 mm), *Asp. sojae* (+41 mm),

Asp. glaucus (+40 mm), *Asp. usamii* (+39 mm), *Asp. awamori* (+41 mm), *Asp. inui* (+40 mm), *Rh. péka* (+41 mm), *M. spinescens* (+39 mm), and *Pen. chrysogenum* (+41 mm) were detected on the anode side with or without tailing effect, those of *Rh. tonkinensis* (−18 mm) and *Rh. javanicus* (−18 mm) were found on the cathode side.

Electrophoretic Behaviors of Saccharogenic Amylases—Electromigration was carried out in the same condition as mentioned above, and the bioautographic technique using *Sacch. cerevisiae* Resse II as the test-organism was employed in this experiment. As shown in Fig. 4, saccharogenic amylases of *Asp. oryzae* and *Asp. sojae* moved towards the anode side (+12 mm and 12 mm) although with lower mobilities than the dextrinogenic amylases of those molds. So far as *Asp. usamii* is concerned, three spots were detected on the anode side (+60 mm, +37 mm, +24 mm), among which two (+60 mm and 24 mm) were saccharogenic amylases without dextrinogenic activity and one (+37 mm) was the mixture of the two enzymes. One component each of the saccharogenic amylases of *Asp. awamori* and *Asp. inui* moved independently from their dextrinogenic amylases towards the anode (+22 mm). But the spots corresponding to saccharogenic amylases of *Rh. tonkinensis* (−40 mm), *Rh. javanicus* (−40 mm), *Rh. péka* (−12 mm), *M. spinescens* (−14 mm),

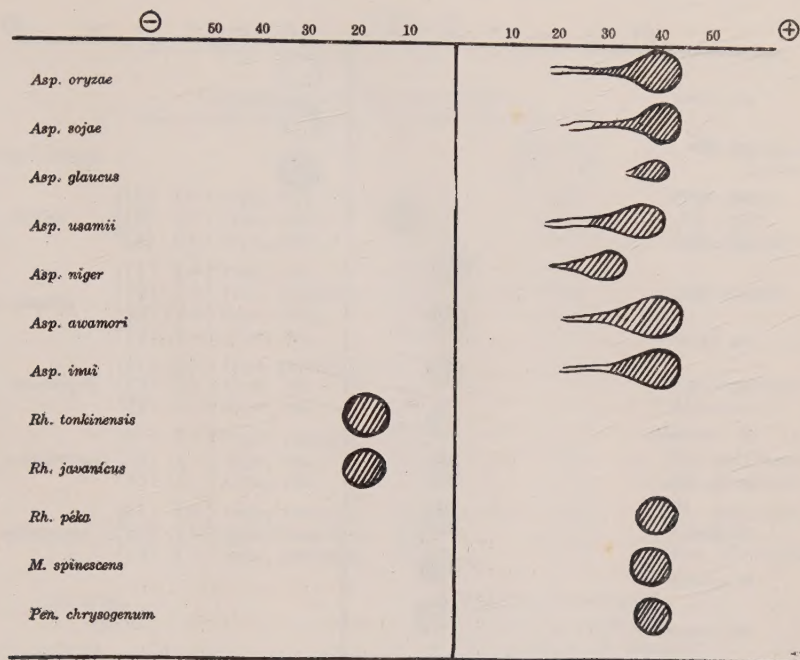


Fig. 3. Electrophoretic Behaviours of Dextrinogenic Amylases (detected by iodine-starch reaction).

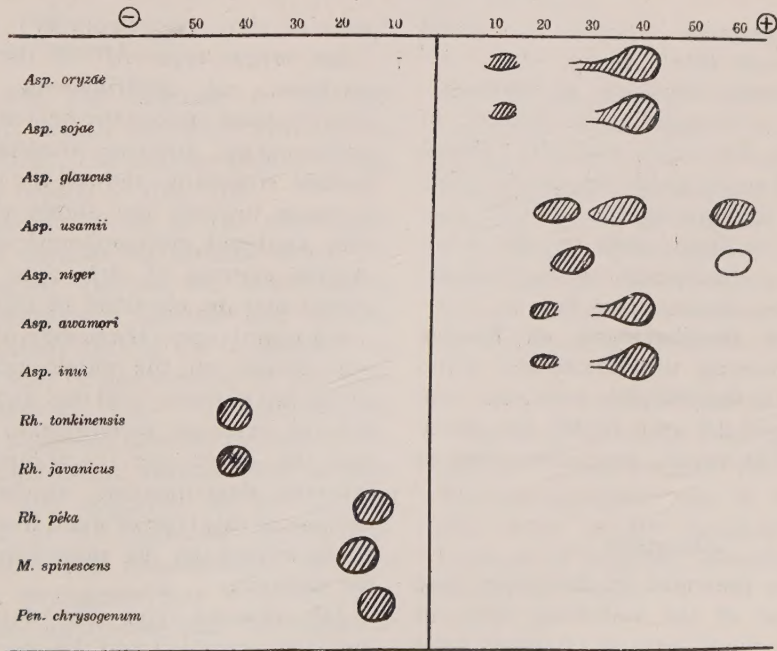


Fig. 4. Electrophoretic Behaviours of Saccharogenic Amylases (detected by bioautography).

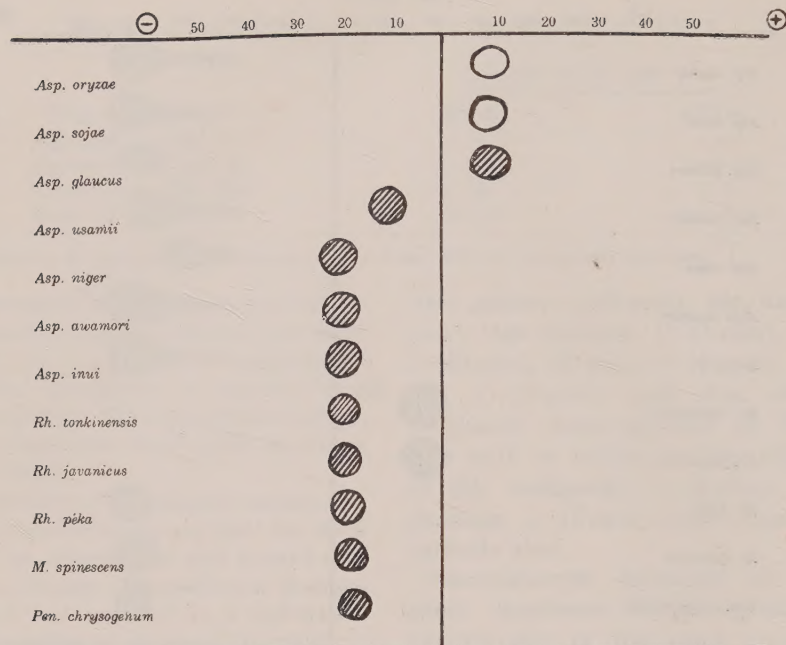


Fig. 5. Electrophoretic Behaviours of Maltases (detected by bioautography).

and *Pen. chrysogenum* (-11mm) were found on the cathode side. (see Fig. 4)

Electrophoretic Behaviors of Maltases—The spots corresponding to maltase of *Asp. oryzae*, *Asp. sojae*, and *Asp. glaucus* were found on the anode side considerably apart from the starting line. On the contrary, the maltase spots of the other strains were discovered on the cathode side (-12~-18 mm). (see Fig. 5)

Effects of Heat-treatment on Enzyme Action—Following the description given by Okazaki, the enzyme solutions were treated at pH 2.5 and at 40° for thirty minutes. The results are summarized in Table IV.

Discussion

The data presented in this paper may be in favor of the following view, in which the mold enzyme systems were classified into five types from enzymologic

point of view. (see Table IV)

Asp. oryzae type—All of the detected amylases, e.g. dextrinogenic amylase (non-resistant against the heat-treatment), saccharogenic amylase (resistant), and maltase (resistant) showed the tendencies to move towards the anode when they were analysed electrophoretically. The enzyme systems of *Asp. sojae* and *Asp. glaucus* may be classified in this type.

Asp. usamii type—Three spots of amylases were found on the anode side, among which two of them (+60 mm and +24mm) may be resistant sacchrogenic amylases, and the other one (+37 mm) a less resistant dextrinogenic amylases. The maltase of this type of mold was definitely characterized by its migration towards the cathode.

Asp. awamori type—The dextrinogenic amylase (resistant) and the saccharogenic amylase (resistant) migrated towards the

Table IV
Classification of Molds by their Enzymatic Constitution.

Typical Strain	Composition of Enzyme System			Mobility (pH 7.6)	Homologous Strains
<i>Asp. oryzae</i>	(1)	(+) type, non-res.,	D. A.	+2.9	<i>Asp. oryzae</i>
	(2)	(+) type, res.,	S. A.	+0.8	<i>Asp. sojae</i>
	(3)	(+) type, res.,	M.	+0.6	<i>Asp. glaucus</i>
<i>Asp. usamii</i>	(1)	(+) type, res.,	S. A.	+4.2	<i>Asp. usamii</i>
	(2)	(+) type, poorly res.,	D. A.	+2.9	
	(3)	(+) type, res.,	S. A.	+1.7	
	(4)	(-) type, res.,	M.	-0.8	
<i>Asp. awamori</i>	(1)	(+) type, poorly res.,	D. A.	+2.9	<i>Asp. awamori</i> <i>Asp. inui</i>
	(2)	(+) type, res.,	S. A.	+1.6	
	(3)	(-) type, res.,	M.	-1.4	
<i>Rh. tonkinensis</i>	(1)	(-) type, non-res.,	D. A.	-1.2	<i>Rh. tonkinensis</i> <i>Rh. javanicus</i>
	(2)	(-) type, res.,	M.	-1.3	
	(3)	(-) type, res.,	S. A.	-2.8	
<i>M. spinescens</i>	(1)	(+) type, non-res.,	D. A.	+2.8	<i>M. spinescens</i>
	(2)	(-) type, non-res.,	S. A.	-0.8	<i>Rh. pèka</i>
	(3)	(-) type, non-res.,	M.	-1.3	<i>Pen. chrysogenum</i>

(+) type	migrated towards anode side
(-) type	migrated towards cathode side
non-res.	non-resistant against the treatment
res.	resistant against the treatment
D. A.	dextrinogenic amylase
S. A.	saccharogenic amylase
M.	maltase
Mobility	cm/V. Sec.

anode side, in contrast to maltase (resistant) which migrated towards the cathode side. The enzyme system of *Asp. inui* may belong to this type.

Rh. tonkinensis—All of the three spots migrated towards the cathode side. The dextrinogenic amylase was non-resistant, but saccharogenic amylase and maltase were resistant against the treatment. *Rh. javanicus* may be one member of this type.

M. spinescens type—The dextrinogenic amylase migrated towards the anode side. The saccharogenic amylase and maltase of this type appeared on the cathode side, not so far from the starting line. All of them were non-resistant. *Rh. pèka* and *Pen. chrysogenum* may be of this type.

It is very interesting that the classifica-

tion presented here shows very good agreement with those of Kitahara and Okazaki in spite of the fact that these three classifications were based on different methods. And the authors believe this work should be evaluated from the view point that the various kinds of diastatic enzyme of molds were successfully separated and became visible on a filter paper.

Acknowledgment

We wish to thank Prof. K. Sakaguchi and Dr. S. Iida for their valuable advice. The test-organisms used in this investigation were kindly supplied from the Institute of Applied Microbiology, University of Tokyo.

Paper Electrophoretic Studies on the Diastatic Enzymes of Molds

Part II. Further Studies on the Properties of the Mold Diastatic Enzymes.

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Received June 26, 1955

The components of mold diastatic enzymes which were successfully separated from one another on a filter paper by the paper electrophoretic technique were extracted with water, and the resulting enzyme solutions were tested with starch and maltose as substrates. By determining the reaction products, the author attempted a speculation on the actual nature of each component enzyme.

In the preceding paper a new technique was described by which component of mold amylase was separated from one another and made visible on a filter paper. This paper is concerned with further studies carried out with the view to study the nature of each component of mold enzymes, and to classify them through the research on hydrolyzed or transformed products of starch and maltose.

Methods

The techniques employed for the preparation of enzyme solutions, electrophoretic migration and detection of enzymes were already given in the preceding paper.

To investigate the nature of each enzyme, the finished paper was cut into several sections so that one section might possibly correspond to one enzyme, with some exceptions where two enzymes be contained in one section. The diastatic enzymes contained in each section were eluted in test-tubes with 1 ml. of distilled water. The aqueous solutions eluted from the sections of the paper strips were employed as enzyme solutions for testing their diastatic activities against starch and maltose.

Starch Solution—High grade soluble starch, dispersed in boiling water and cooled to room

temperature, was used as the substrate.

Maltose Solution—To eliminate glucose and other impurities, reagent grade maltose was treated with active carbon and recrystallized. Paper chromatographic examination showed that the preparation was free from other reducing sugars.

Digestion of Starch—Mixture of 2 ml. of the starch solution (2%) and 0.3 ml. of the enzyme solution were placed in an incubator (30°), after adjusting the pH to 4.8~5.2 with 0.5 M phosphate buffer.

Digestion of Maltose—Mixtures of 2 ml. of the maltose solution (3%) and 0.3 ml. of the enzyme solution were kept in an incubator in the same manner as described above.

Enzyme action was stopped after 15~20 hours by heating the digests at 100° for five minutes, and the resulting reducing sugars were examined by the paper chromatographic technique.

Paper Chromatography—The paper chromatographic technique described by A. Jeanes et al¹⁾ was used for the identification of reducing sugars. About 0.01 ml. aliquot was placed on a filter paper and developed with *n*-butanol-pyridine-water mixture (3:2:1.5 by volumes) by the multiple ascent technique.

The finished chromatogram was sprayed with ammoniacal silver nitrate reagent (5%), and heated at 100° for a few minutes. The areas corresponding to reducing sugars appeared in

1) A. Jeanes, C. F. Wise and R. J. Dimler, *Anal. Chem.* **23**, 415 (1951).

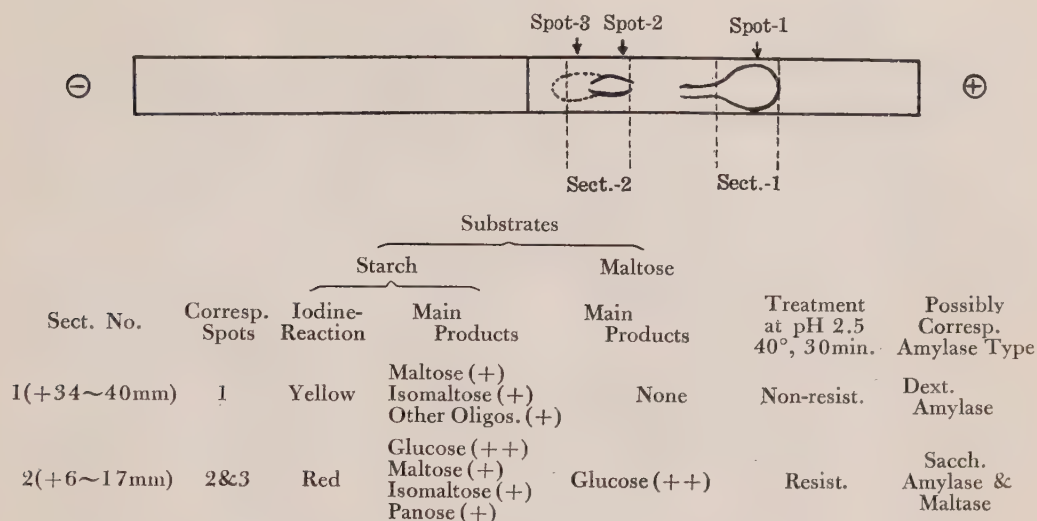


Fig. 1. Digestion of Starch and Maltose by *Asp. oryzae* Amylases.

brown-black on the paper.

Results and Discussion

The enzyme system of the following five species, representing five mold types described in the preceding paper, were examined in this experiment,

Aspergillus oryzae (0-8-1), *Asp. usamii* (1-1), *Asp. awamori* (K-2 0913), *Rhizopus tonkinensis* (R-64), *Mucor spinescens* (Mu-3).

The data obtained are illustrated in Figs. 1~5. The nature of each enzyme was argued depending on the following definitions;

(1) Dextrinogenic amylase—An enzyme responsible for the production of comparatively high molecular dextrans from starch, which develop yellow color with iodine and permit little or no growth of *Saccharomyces cerevisiae* Rasse II when bioautographically tested (cf. preceding paper). No production of glucose from maltose.

(2) Saccharogenic amylase—An enzyme responsible for the production of

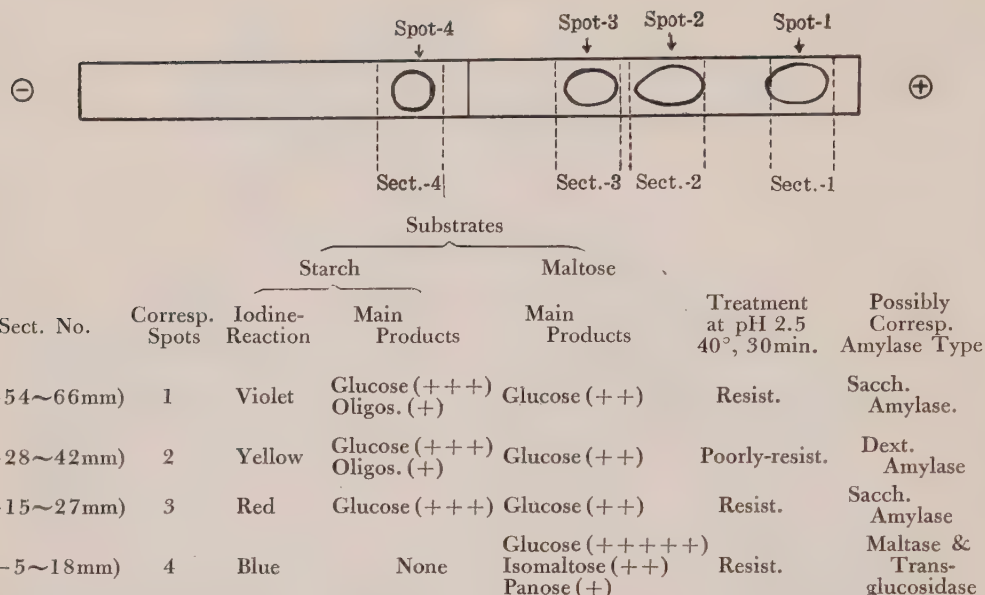
comparatively low molecular sugars from starch, which develop red color with iodine and permit the growth of *Sacch. cerevisiae* Rasse II when bioautographically tested. This term, of course, includes the so-called glucogenic amylase.

(3) Maltase—An enzyme responsible for the production of glucose from maltose and giving no degradation product from starch.

(4) Transglucosidase—An enzyme responsible for the production of isomaltose, panose and other oligosaccharides from maltose.

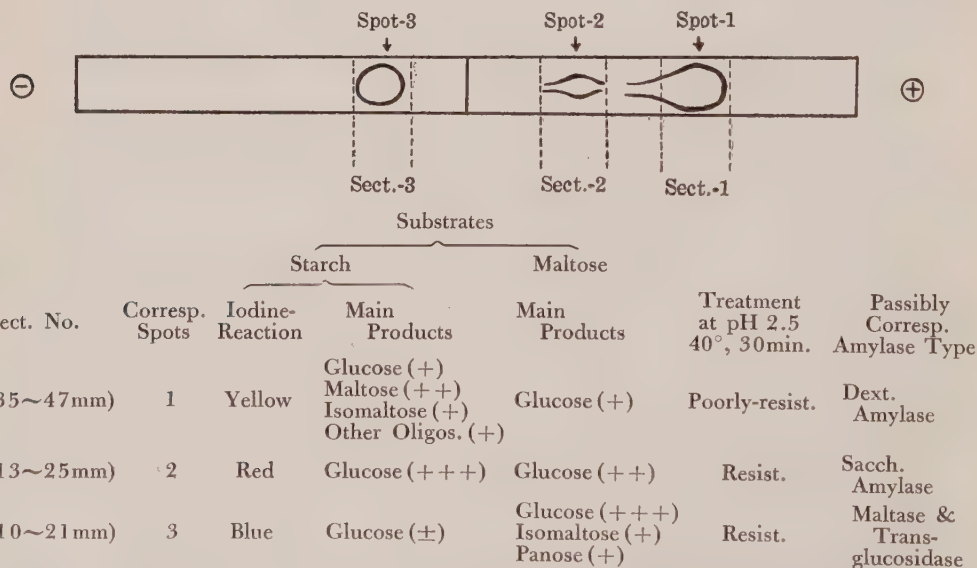
Asp. oryzae—As shown in Fig. 1 the enzyme in sect.-1 was able to convert starch into some oligosaccharides such as maltose, isomaltose, and others, an evidence indicating that spot-1 might correspond to dextrinogenic amylase. Sect.-2 (including spots-2 and 3) produced glucose, maltose, isomaltose, and panose from starch, and glucose from maltose. This section might correspond to saccharogenic amylase and maltase.

Asp. usamii—The results are presented

Fig. 2. Digestion of Starch and Maltose by *Asp. usamii* Amylases.

in Fig. 2. Each extract of these three sections (sects.-1, 2, and 3) produced glucose from starch as well as from maltose, although sect.-2 (corresponding to spot-2)

gave yellow color by iodine solution and seemed less-resistant against the treatment (pH 2.5, 40°, 30 minutes). Both spots-1 and 3 might correspond to saccharogenic

Fig. 3. Digestion of Starch and Maltose by *Asp. awamori* Amylases.

amylases, and spot-2 might correspond to dextrinogenic amylase. As to the forth section, glucose, isomaltose and panose were produced from maltose, this spot therefore might have the activities of transglucosidase in addition to maltase.

Asp. awamori—As seen in Fig. 3 by the addition of sect.-1 extract, starch was converted to a series of low molecular weight sugars such as glucose, maltose

and other oligosaccharides, and maltose was converted to glucose, indicating the possibility that this spot might correspond to dextrinogenic amylase and maltase. Because sect.-2 produced only glucose from both starch and maltose, the corresponding spot might be glucose-forming saccharogenic amylase. The maltase spot in sect.-3 also showed transglucosidic action, converting maltose into other oli-

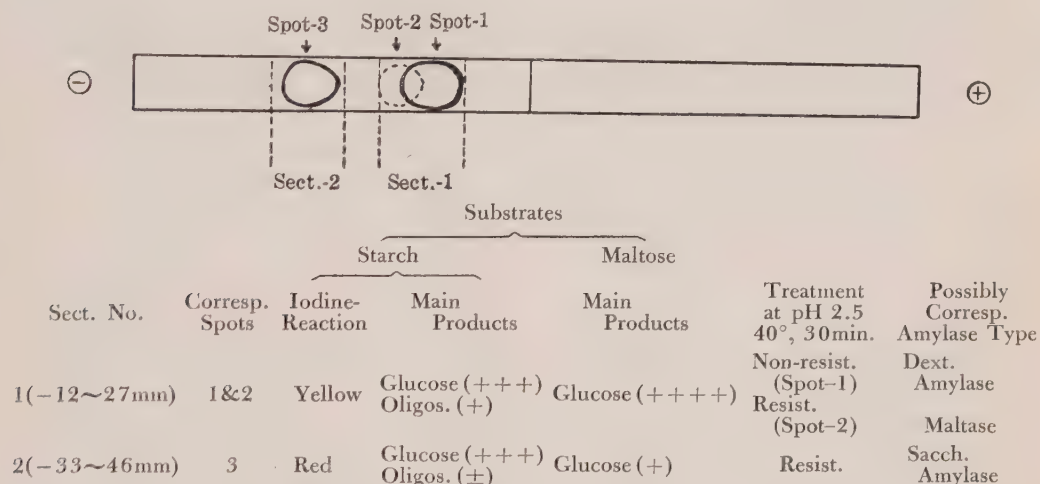


Fig. 4. Digestion of Starch and Maltose by *Rh. tonkinensis* Amylases.

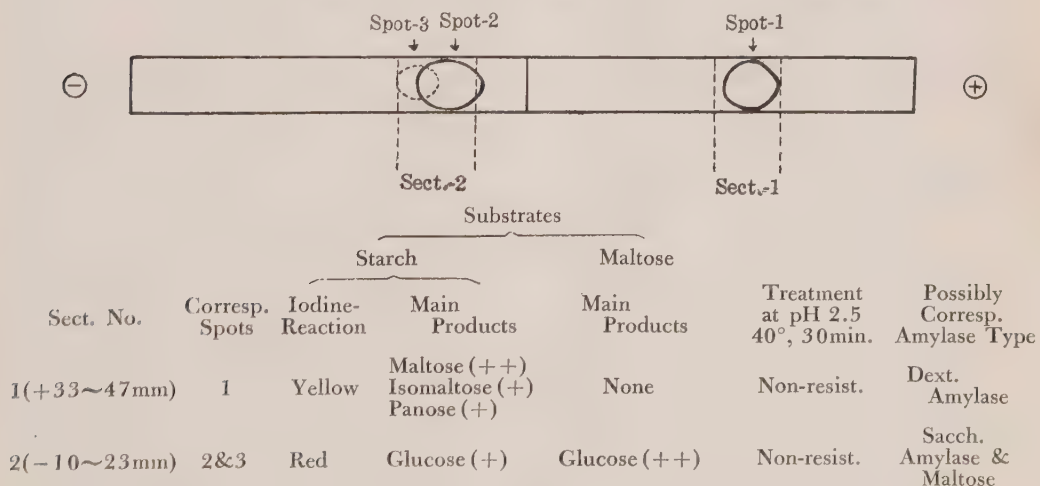


Fig. 5. Digestion of Starch and Maltose By *M. spinescens* Amylases.

gosaccharides such as isomaltose and panose.

Rh. tonkinensis—The results are shown in Fig. 4. The spots-1 and 2 were found at the adjacent position, but spot-1 being less-resistant against the heat-treatment. Therefore it may be said that the dextrinogenic amylase and maltase of this mold were found in separate spot. Sect.-3 (spot-3) seemed to be glucose-forming saccharogenic amylase.

M. Spinescens—As shown in Fig. 5 a series of sugars including maltose, isomaltose, and panose were produced from starch by an aqueous extract of sect.-1

(spot-1), showing the possibility of being dextrinogenic amylase. Sect.-2 (including spots-2 and 3) seemed to be a mixture of saccharogenic amylase and maltase, although further separation of these two spots was as yet unsuccessful.

Acknowledgments

I wish to thank Prof. K. Sakaguchi, Dr. S. Iida and Dr. Y. Ikeda for their valuable advice. The test-organisms used in this investigation were kindly supplied from the Institute of Applied Microbiology, University of Tokyo.

Studies on the Metabolism of D-amino acid in Microorganisms.

Part. I. Degradation of D-glutamic acid in Genus *Aerobacter*.

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Received July 29, 1955

Several strains of bacteria belonging to genus *Aerobacter* were found to oxidize D-glutamate rapidly, while they show feeble oxidative activity toward the L-form even when they were grown in the medium containing DL-glutamate.

The isolation of L-glutamate, a natural amino acid, from its DL-form was achieved by the degradation of D-glutamic acid using one of these strains.

This may be the first observation on a natural amino acid obtained from the racemic one by the metabolic action of the organism.

A new enzyme, D-glutamic acid oxidase, which is responsible for D-glutamate degradation in this organism and differs from Krebs' D-amino acid oxidase, has been postulated.

Non-specific D-amino acid oxidase has been found in mammalian tissues¹⁾, fungi^{2,3)}, and bacteria⁴⁾. D-Glutamic acid, however, is not or even slowly oxidized by these enzyme preparation. The specific D-aspartic acid oxidase found by Still et al.⁵⁾ was reported to act weakly on D-glutamic acid. Therefore, not much is known so far, on the metabolic behavior of D-glutamic acid. During the search for the microorganism, which can grow on the medium containing D-glutamic acid as a sole source of carbon and nitrogen, it is found that several isolated strains belonging to genus *Aerobacter* can metabolize D-glutamic acid

more rapidly than the L-form even when the organisms are grown in DL-glutamic acid. It is possible to obtain L-glutamic acid from the DL-form by decomposing D-glutamic acid using one of these strains.

Since D-amino acid has been regarded as an unnatural form, it is a very interesting fact that the natural L-form amino acid is obtained from the DL-form by the metabolic action of microorganisms.

Methods and Materials

1. Substrates: D-, L-, and DL-Glutamic acid and α -ketoglutaric acid used in the following experiments were kindly supplied by Ajinomoto Co. Ltd.

2. Preparation of the intact cells: Bacteria isolated from soil according to the methods described in experimental were grown in the usual broth-agar and stocked in such a way. Resting cells were prepared by inoculating the bacterium in medium A which has the following composition, i.e., D-glutamic acid 1.0%, K_2HPO_4 0.2%, tap water, and the pH was adjusted to

* Institute of Applied Microbiology, University of Tokyo.

1) H. A. Krebs: *Z. Physiol. Chem.*, **217**, 191 (1933).

2) N. H. Horowitz: *J. Biol. Chem.*, **154**, 141 (1944).

3) R. L. Emerson, M. Puziss, and S. G. Knight: *Arch. Biochem.*, **25**, 299 (1950).

4) P. K. Stumpf and D. E. Green: *Federation Proc.*, **5**, 157 (1946).

5) J. L. Still, H. V. Buell, W. E. Knox, and D. E. Green: *J. Biol. Chem.*, **179**, 831 (1949).

7.2. In some experiments medium B, containing 1% of DL-glutamic acid in place of the D-form in medium A was used. In every case, cells were grown aerobically on the shaking machine at 30°C. After incubation, the cells were harvested by centrifuging, washed twice with water or saline, and finally suspended in water. The weight of the cells in suspension was determined by drying them at 110°C for several hours.

3. Analytical methods: The oxidative activities of the resting cells towards various substances were estimated manometrically in Warburg vessels at 30°C. L-Glutamic acid was assayed by the manometric method according to E. F. Gale et al.⁶⁾ using L-glutamic acid decarboxylase preparation from *E. coli* crooks strain. D-Glutamic acid was detected by paper partition chromatography after the enzymatic degradation of the L-form by the decarboxylase. The detection of nonvolatile and keto acids were carried out by paper chromatography and the quantitative estimation of α -ketoglutarate was made according to the methods of Friedemann and Haugen⁷⁾.

Experimental

1. Isolation of the bacteria which can decompose D-glutamic acid.

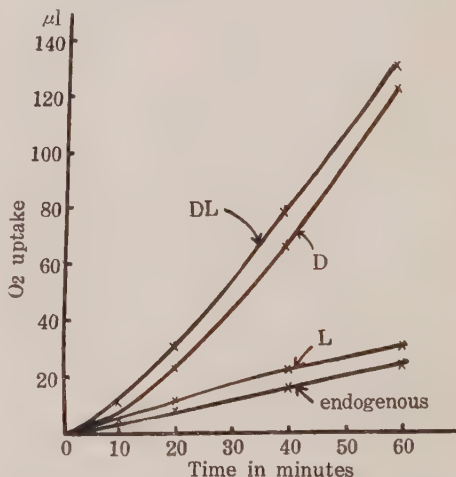
For the purpose of obtaining the organism which can metabolize D-glutamic acid, various samples such as soil or swage gathered from the district of Tokyo were put into the enrichment medium containing 0.5% of D-glutamic acid, 0.2% of K_2HPO_4 and tap water. The reaction of the enrichment medium was adjusted to pH 7.2. After standing for 2 or 3 days at 30°C, the organisms grown in the medium were inoculated to the new enrichment medium and stored at 30°C for 2 or 3 days. Almost one hundred strains of bacteria were isolated by the usual method from the above culture. The rates of growth of these strains in the medium containing D-glutamic acid was compared with those in the medium which contained L-glutamic acid as a sole source of carbon and nitrogen. The

majority of the isolated strain showed similar growth rate in either D-or L-glutamic acid medium or even grew better in the L-glutamate medium. Several strains, however, showed heavy growth in the D-glutamate medium while only feeble growth was observed in the L-glutamate one. Biochemical and morphological examinations of these extraordinary strains revealed that they belonged to the genus *Aerobacter*. The following experiments were carried out using *Aerobacter* strain A, one of these strains.

2. Oxidative activities of resting cells towards D-, L-, and DL-glutamic acids.

a) Cells grown in D-glutamate

Aerobacter strain A was grown aerobically in the medium A for 22 hrs. The oxidative activities of resting cells towards D-, L-, and DL-glutamic acids were estimated manometrically. The rate of oxidation of D-glutamic acid was about twenty times greater than that of L-glutamic acid as shown in Fig. 1, and Table I. Since these organisms were grown in the D-glutamate medium, it may be considered reasonable that they showed



Each vessel contained cell suspension 0.4 ml. (1.2 mg dry weight), M/15 phosphate buffer pH 7.2 1.0 ml., and $3.3 \mu M$ of D-, L-glutamic acid $6.6 \mu M$ of DL-glutamic acid, 0.2 ml. 0.2 ml. of 20% KOH was placed in the center well.

Incubation was carried out at 30°C in aerobic condition.

Fig. 1. Oxidation of Glutamic Acid by the Cells grown in D-Glutamic Acid.

6) E. S. Taylor, E. F. Gale, *Biochem J.*, **39**, 52, (1945).

7) T. E. Friedemann and G. E. Haugen: *J. Biol. Chem.*, **147**, 415 (1943).

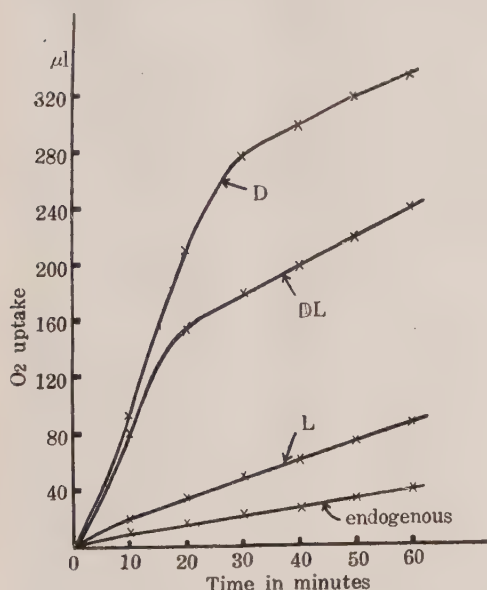
Table I
Oxidative Activities of Strain A toward
Glutamic Acid.

Substrate used	D	L	DL
QO ₂	71	3	79

stronger oxidative activity towards D-glutamate than towards the L-form.

b) *Cells grown in DL-glutamate.*

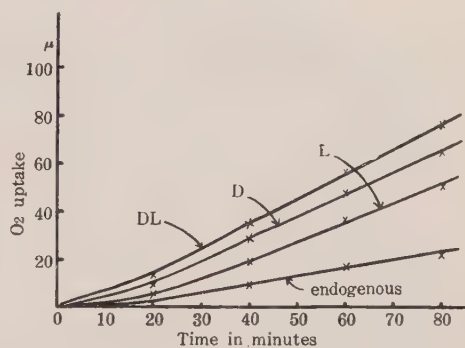
Aerobacter strain A in the medium B, grew just as rapidly as in the medium A. It is interesting to examine the rate of oxidation of D-, L-, and DL-glutamic acids by the cells grown in DL-glutamate. Experiments were carried out and the typical results are presented in Fig. 2, and Fig. 3, and Table II. As it is shown, D-glutamate was oxidized some ten times faster than the L-



Each vessel contained cell suspension 0.5 ml. (4 mg dry weight), M/15 phosphate buffer pH 7.2 1.0 ml., and 6.6 μM of glutamate in a total volume of 1.7 ml.; 0.2 ml. of 20% KOH was placed in the center well.

Fig. 2. Oxidation of Glutamic Acid by the Cells grown for 24 hrs. in DL-Glutamic Acid.

form by the 24 hours culture, the activity, however, being decreased greatly in the cells obtained at 42 hours culture. It must be mentioned that no D-glutamate was found in the 42 hrs.



Each vessel contained cell suspension 0.5 ml. (2 mg dry weight), M/15 phosphate buffer pH 7.2 1.0 ml., and 6.6 μM of glutamate in a total volume of 1.7 ml.; 0.2 ml. of 20% KOH was placed in the center well.

Fig. 3. Oxidation of Glutamic Acid by the Cells grown for 43 hrs. in DL-Glutamic Acid.

Table II
Oxidative Activity of Strain A toward Glutamate.

Substrate used	D	L	DL
QO ₂			
24 hr.	171	15	101
43 hr.	16	10	21

incubation medium.

c) *Cells grown in broth with or without DL-glutamate.*

The growth rate of the bacterium was remarkably higher in the usual broth than in mediums A and B. The metabolic activities towards D, L- and DL-glutamic acids by the cells grown in broth with or without DL-glutamic acid were examined. Appreciable activity toward the D-glutamic acid can not be detected in the cells grown in the broth medium and the activity of the cells grown in broth with 0.5% DL-glutamate toward D-glutamic acid was as weak as that toward the L-form. The results are presented in Table III.

d) *Cells grown in DL-glutamate medium added with yeast extracts.*

The addition of 1% of yeast extracts to medium B favors the growth of the organism greatly. The rate of oxidation by these cells were examined and it was found that the addition of 1% of yeast extracts resulted no decrease in the oxidative activity of D-glutamate, as shown in Table

Table III
Oxidative Activities by the Cells grown in Broth with or without DL-Glutamate.

Substrate used Medium	D-glutamic acid	L-glutamate	DL-glutamate
Broth	2	3	3
Broth+0.5% DL-glutamate	31	28	59

Each vessel contained cell suspension 0.5 ml. (2-5 mg dry weight), M/15 phosphate buffer pH 7.2 1.0-1.5 ml., and 6.6 μ M of DL-glutamate 3.3 μ M of D- or L-glutamate in a total volume of 1.7-2.2 ml.; 0.2 ml. of 20% KOH was placed in the center well.

Table IV
Oxidative Activities by the Cells grown in DL-Glutamate with Yeast Extract.

Substrate used	D-glutamate	L-glutamate	DL-glutamate
QO ₂	147	15	97

Each vessel contained cell suspension 0.5 ml. (2 mg dry weight), M/15 phosphate buffer pH 7.2 1.0 ml. and 6.6 μ M of glutamate in a total volume of 1.7 ml.; 0.2 ml. of 20% KOH was placed in the center well.

IV. From the preceeding experiments, it is clear that the activity of D-glutamate oxidation occurs only in the cells grown in the medium containing D-glutamate. The experiments showed that the addition of L-glutamate to the medium has little influence on the activity of D-glutamate oxidation. It is probable, therefore, that the enzyme responsible for the oxidation of D-glutamate is adaptive in nature. It was also found that the cells of *Aerobacter aerogenes* ATCC 9621 grown in the same conditions, oxidized D-glutamic acid about twice as fast as L-glutamic acid.

e) *Adaptation of resting cells to D-glutamate.*

In order to clarify the mechanism of adaptation to D-glutamate more precisely, it is preferable to adapt the resting cells to D-glutamate. As stated previously, the cells grown in the usual broth can not oxidize D-glutamate. The oxidative activity towards D-glutamate could not be increased appreciably even when the cells were incubated aerobically with D-glutamic acid as long as 3

hours. Furthermore, the cells grown in the broth containing D-glutamic acid and which afterwards decreased their activity toward D-glutamate, were found incapable of restoring the activity to D-glutamate within an hour. No indication, therefore, is available for the adaptation of resting cells to D-glutamic acid.

3. Oxidative activities towards other amino acids.

Since it is possible assumed that the oxidation of D-glutamic acid might be catalyzed by a non-specific D-amino acid oxidase, the oxidative activities of the resting cells towards various amino acids must be examined. It was revealed in an earlier experiment, that strain A could grow neither in a medium containing DL-methionine, DL-valine, nor DL-isoleucine as a sole source of carbon and nitrogen. The organism, however, showed sluggish growth in DL-aspartic acid. The cells grown in DL-glutamate could hardly oxidize

Table V
Oxidative Activities towards other Amino Acids by the Cells grown in DL-Glutamate.

Substrate used	D-glutamate	DL-aspartic acid	DL-methionine
QO ₂	101	19	6

Each vessel contained cell suspension 0.4 ml. (2 mg dry weight), M/15 phosphate buffer pH 7.2 1.0 ml., 10 μ M of D-glutamate, 20 μ M of DL-aspartate and DL-methionine respectively in a total volume of 2.0 ml. of 20% KOH was placed in the center well.

DL-methionine, while they oxidized DL-aspartic acid but in a rate lower than D-glutamic acid. The results are given in Table V. It had been shown by Krebs et al. that non-specific D-amino acid oxidase decomposes DL-methionine rapidly, but can scarcely oxidize DL-glutamic acid. From these considerations, the results obtained by the authors may indicate the presence of a new enzyme—a specific D-glutamic acid oxidase. There remains, however, some objection for the above conclusion, because it is also possible that D-methionine will penetrate the cell-wall less easily.

4. Analysis of the incubation medium.

a) *D-glutamic acid as a substrate.*

When the organism was grown in medium A which contained D-glutamate, a considerable amount of L-glutamic acid is formed in the incubation medium. In a typical experiment, when the duration of incubation was 45 hrs., 0.72mM of L-glutamic acid corresponding to about 11% of the added D-glutamate was found. This finding may be explained either by the glutamic racemase or the synthesis of L-glutamic acid from the D-form. The latter hypothesis seems to be probable, but this will be discussed later.

b) *DL-glutamic acid as a substrate.*

As predicted from the manometric data, D-glutamic acid in the medium B decreased rapidly, while L-glutamic acid did not. Therefore, it is possible to obtain L-glutamate from the DL-form through decomposition of D-glutamate by the bacterium. Three hundred ml. of medium B was inoculated with the organism, and incubated aerobically for 43hrs. at 34°C. After incubation, the cells were removed by centrifugation. The L-glutamate estimated in the supernatant solution was 9.7mM which was almost identical with the initial amount of L-glutamic acid (10.5mM). No D-glutamic acid could be detected. The solution was concentrated in vacuum and 1g. of crude crystals of L-glutamic acid was obtained. This was recrystallized three times from water and alcohol and 700 mg. (4.8mM) of pure L-glutamic acid, which gave the following analytical values, was obtained.

mp.	206–207°C,
total N.	9.53%,
$[\alpha]_D^{25} = +31.2 \pm 0.4.$	

Since the organism has some activity toward the L-glutamate, it also slowly decreases if the incubation is continued after disappearance of the D-form from the medium. Noteworthy is the fact that in some experiments, a larger amount of L-glutamate than the amount initially added was detected in the metabolized medium, where little or no D-form could be found.

c) *Decomposition products of DL-glutamic acid other than L-glutamic acid.*

About 2 molecules of oxygen were taken up per each molecule of D-glutamic acid consumed by the organism. If the assumption that the oxidation of the D-glutamic acid primarily gives rise to α -ketoglutarate and further oxidation of α -ketoglutarate is followed via the Krebs' citric acid cycle is possible, the oxidation must proceed to the pyruvate stage. This view, however, is not supported because a very small amount of pyruvate as a decomposition product of D-glutamate is found. Since it is well known that the substrate oxidized is to some extent assimilated to the cell material, it will be impossible to determine the mechanism of D-glutamate oxidation from the amounts of oxygen consumed. In order to make the oxidative pathway of D-glutamate degradation clear, further researches for the intermediates of D-glutamate oxidation were carried out. Strain A was grown in 100ml. of medium B by the stationary culture for 10 days at 30°C. The metabolized medium was centrifuged to remove the cells. The supernatant solution was acidified and continuously extracted with ether. Non-volatile organic and keto acids extracted with ether were assayed by paper partition chromatography. A well defined spot of pyruvic acid was found and further, two spots of non-volatile organic acids were also found, one corresponding to succinic acid and the other to pyrrolidone-carbonic acids. The latter compound does not seem to be the enzymatic products from DL-glutamic acid because it is formed from the glutamate by mere heating and acidification. Probably, it might be formed during the ether extraction procedure. A postulated intermediate for the oxidation of D-glutamate is α -ketoglutarate. Since it is well known that arsenite inhibits the oxidation of α -ketoglutarate, the effect of arsenite on the oxidation of D-glutamate by the strain A

Table VI

Substrate used	D-glutamate	D-glutamate + 10^{-3} M Arsenite	D-glutamate + 5×10^{-4} M Arsenite
QO ₂	101	9	36
Inhibition		91%	65%

Each vessel contained cell suspension 0.4 ml. (0.8 mg dry weight) M/15 phosphate buffer pH 7.2 1.0 ml, and 10^{-4} M of D-glutamate in a total volume of 2.0 ml. of 20% KOH was placed in the center well.

was examined. Arsenite in the concentration of 10^{-3} M blocked the oxidation of D-glutamate almost completely, but when the concentration was decreased to 5×10^{-4} M, the inhibition of D-glutamate oxidation also decreased to 65%, as shown in Table VI. In these concentrations of arsenite, α -ketoglutarate oxidation by the bacterium was almost completely inhibited. If the α -ketoglutarate oxidase system was more sensitive to arsenite than the D-glutamic acid oxidase system accumulation of α -ketoglutarate in the presence of arsenite might be expected. This experiment was carried out, and it was found to be the case.

Strain A was inoculated to the 100 ml. of medium B containing M/2000 arsenite. Incubation was done at 30°C for 45 hrs. After incubation the medium was centrifuged and the supernatant solution was treated with 2,4-dinitrophenylhydrazine HCl solution. The yellowish precipitate formed was filtered off, and it was purified according to the method of Krebs. About 20 mg. of pure crystal were obtained, which decomposed at 216–217°C, and gave a well defined spot corresponding to α -ketoglutarate hydrazone by paper partition chromatography. Since no D-glutamate was detected in the medium and remained almost the theoretical amount of L-glutamate there, it may be concluded that α -ketoglutarate is the primary degradation product of the D-glutamate.

Discussion

The strong oxidative activity of strain A toward D-glutamic acid is produced when the strain was cultured aerobically in the medium containing D- or DL-glutamic acid, and decreased rapidly as soon as D-glutamic acid was consumed; whereas the cells grown in broth could hardly

oxidize D-glutamic acid. These results may indicate that D-glutamic acid oxidase system involved an induced enzyme. Enzymatic adaptation to D-glutamate of the resting cells grown in the broth were carried out unsuccessfully. Probably, some unknown conditions for the adaptation must be required.

The metabolic pathway of the amino acid oxidation has been pursued in various laboratories, and it can be considered that the following four enzyme systems are known for the metabolism of amino acid; amino acid oxidase, transaminase, racemase, and decarboxylase. From consideration of these the following hypothesis for the primary degradation of D-glutamate in strain A are possible. First, D-glutamate is oxidized by either a specific or a non-specific oxidase to form α -ketoglutarate and ammonia. Second, the amino group of the D-glutamate is transferred to an other keto acid to form a new amino acid and α -ketoglutarate. The third hypothesis is that the D-glutamate is racemized by the glutamic racemase to form DL-glutamate, and L-glutamic acid is oxidized by the well known L-glutamic acid dehydrogenase. The last possibility is that D-glutamate is metabolized through any other route than via α -ketoglutarate, as for example through the decarboxylation by D-glutamic acid decarboxylase. In the experimental process an appreciable amount of the L-glutamate is found to be formed

by the metabolism on D-glutamic acid. This may provide evidence for the occurrence of either racemase, transaminase, or the asymmetric synthesis of the L-form from the D-glutamate. As it has been shown in the Experiment 4, larger amounts of L-glutamate than D-glutamate remain in the incubation medium. Since it was shown by many workers that amino acid racemase can act on both D- and L-amino acid, the third hypothesis may be excluded. The last possibility must be also excluded because carbon dioxide is not remarkably evolved in the anaerobic decomposition of the D-glutamate. The first hypothesis therefore seems to be most probable as the main oxidative pathway for the break down of D-glutamic acid, because α -ketoglutarate is formed as a decomposition product of D-glutamate in the presence of arsenite. The fact that the D-glutamate is most rapidly oxidized by the cells grown in the DL-glutamate strongly supports this view. Hence, the question "whether the oxidase responsible for D-glutamate degradation is specific or not?" may arise. As it was shown in Experiment 3, D-methionine, one of the suitable substrates for nonspecific D-amino acid oxidase, was proved to be scarcely oxidized by the resting cells. Furthermore, the only D-amino acids which are oxidized at detectable rates by the organism were D-glutamic and D-aspartic acid. These amino acids have been proved to be slowly oxidized by nonspecific D-amino acid oxidase. Since specific D-aspartic acid oxidase was reported by Still, and

crude D-aspartic acid oxidase was found to act slowly on D-glutamate, it is possible to assume that the D-aspartic acid oxidase plays an important role in the metabolism on D-glutamate. The rate of oxidation of D-aspartate in this organism, however, is so small as compared with that of D-glutamate. These observations strongly suggest the occurrence of specific D-glutamic acid oxidase. Recently, specific D-glutamic acid oxidase is also found in fungi by the authors. The crude enzyme preparation extracted from the fungus mycelium is found, to act on D-aspartic acid. The nature of the specific D-glutamic acid oxidase of fungi will be reported in the near future.

The authors are indebted to Ajinomoto Co. Ltd. for analysis and supplying the necessary samples in this study.

Summary

Oxidative metabolism of D-glutamate by microorganism was investigated. It was found that several isolated strains of bacteria belonging to the genus *Aerobacter* could metabolize D-glutamate more rapidly than the L-form even when they were grown in the DL-glutamate medium. Pure L-glutamic acid could be successfully obtained from the DL-form by decomposing D-glutamic acid using one of these strains. From several experimental results, the occurrence of a specific oxidase responsible for the oxidation of D-glutamic acid was suggested.

Fat Synthesis in Unicellular Algae.

Part I. Culture Conditions for Fat Accumulation in *Chlorella* Cells.

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The cultural conditions of *Chlorella ellipsoidea* were studied with special reference to lipide accumulation. It was ascertained that there is a close relation between the lipide content and the nitrogen content of cells. Cultivation with the nitrogen-free medium was applied to produce nitrogen deficient cells, and in this culture were studied the effects of light intensity, algal density and depth of algal suspension upon lipide accumulation. The annual yield of fat by mass-culture was roughly estimated.

The first investigation on the production of fat by the cultivation of unicellular algae was made in Germany during the last War, using diatom as the test organism. After the War, Spoehr and Milner¹⁾ found that *Chlorella pyrenoidosa* accumulates considerable amounts of fat in its body grown in the nitrogen deficient medium. At that time *Chlorella* was attracting world-wide attention as a new source of protein, and consequently it was also noticed as a fat producer and some discussions were made^{2, 3)}.

Fogg and Collyer⁴⁾ examined the characteristics of several species of green algae, and blue-green algae, including *Ch. vulgaris*, with reference to the accumulation of fat, and demonstrated that *Ch. pyrenoidosa* has overwhelming ability to accumulate fat, compared with all the other algae examined. The authors also examined *Ch. ellipsoidea* and obtained almost the same results as *Ch. pyrenoidosa*. And then some quantitative

investigations on the fat accumulation of *Ch. ellipsoidea* were pursued, while no quantitative investigation concerning the relation between the degree of fat accumulation and the light intensity or other environmental conditions had ever been made.

Experimental methods

The culture apparatus is shown in Fig. 1. The culture flask was a flat bottle of 3 cm thickness and of 700 ml. capacity, which was originally used by Tamiya et al⁵⁾. The culture bottle was immersed in a glass water bath to be maintained at 25°C, and was illuminated with a 300 W or 500 W flood lamp on one side from the outside of the water bath. The light intensity was regulated by changing the voltage or the distance between the lamp and the bottle. The illuminated area per 500 ml. medium in the bottle was 170 cm². In the initial stage of the experiments, four fluorescent lumps (white) per one side of the bottle were used instead of a flood lamp.

Five to four percent CO₂ rich air was used in aeration and the aeration rate was 500 ml. per 500 ml. medium per minute.

The composition of the basal medium was as follows :

KNO₃ 0.025 mol/l
MgSO₄·7H₂O, KH₂PO₄ 0.01 mol/l

5) H. Tamiya, et al, *ibid*, Chap. 16, p. 204.

1) H. A. Spoehr and H. W. Milner, *Plant Physiol.*, **24**, 120 (1949).

2) F. Gummert, *Fette u. Seifen*, **52**, 453 (1950).

3) H. W. Milner, *J. Am. Oil Chem. Soc.*, **28**, 363 (1951).

4) G. E. Fogg and D. M. Collyer, "Algal Culture", Carnegie Inst. Washington Pub. 1953, Chap. 12, p. 177.

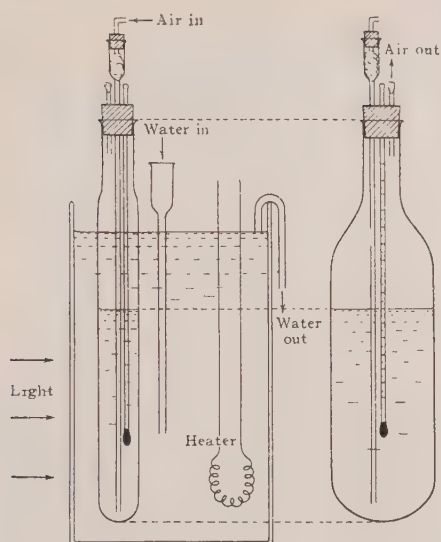


Fig. 1. Culture Apparatus.

Micro-elements*

Fe 10 ppm; B, Zn 4 ppm; Mn,
 Mo 0.8 ppm; Cu, Co 0.2 ppm
 Ethylenediamine tetraacetic acid 0.1g/l
 pH 5.5

When the nitrogen-free medium was used, the nitrogen source was just omitted from the basal medium.

Algal concentration was measured by the dry weight of algae in 10 ml. of algal suspension, drying for 24 hrs. at 100°C after centrifuging and washing twice by distilled water.

Semi-micro Kjeldahl method was used for nitrogen determination, and Haskin's method⁶⁾ was applied to chlorophyll determination. Crude fat was determined by ether extraction. Using algal cells kept in a vacuum desiccator after dried on a tonplate, ether-extraction was carried out several times, inserting the process of grinding the algal cells in a mortar with emery-powder. This procedure is necessary for complete extraction.

Results and Discussions

Milner⁷⁾ once obtained *Chlorella* cells

* The following compounds were used: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, H_3BO_3 , $\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$.

6) H. H. Haskin, *J. Biol. Chem.*, **144**, 149 (1942).

7) H. W. Milner, *J. Biol. Chem.*, **176**, 813 (1948).

which contained lipides in 20–85% of dry matter. In this case, in order to obtain the *Chlorella* cells containing lipides in 75.5% of dry matter, he used a culture medium of low nitrogen concentration (N: 0.0025 mol/l, equivalent to the algal density 0.5 g/l, containing the total nitrogen in 7% of dry matter), and cultivated in Fernbach flasks, illuminating them from the bottom with 200 W tungsten lumps, for 75 days. The final algal concentration was reported to be 2.5 g/l and the total nitrogen of the cells was 1.23%. The normal *Chlorella* cells which can be obtained by culturing in a medium containing sufficient nitrogen source, contain total nitrogen in 7–8% of dry matter. So it is clear that the cells of high lipid content are of low nitrogen content compared with the normal cells.

The authors also tried to obtain nitrogen deficient cells by culturing in the media of diverse degrees of nitrogen deficiency, starting cultivation with a very small inoculum size. The result is shown in Table I. From this result, it is quite conceivable that *Chlorella* can grown in a completely-nitrogen-free medium and accumulates lipid during the growth. And it is also quite possible that the initial algal concentration, the depth of the medium and the light intensity will have influence on the final degree of nitrogen deficiency of the cells. Milner³⁾ reported that he had never obtained *Chlorella* cells of lipid content higher than 30% when residual nitrogen in the medium was more than 0.001 mol/l. the authors pursued the following experiments, having cultivated in a nitrogen-free medium with the inoculum of washed normal cells which had been cultivated in advance in the basal medium. As the result, it is quite clear that *Chlorella* can

Table I
Effect of the Nitrogen Source Concentration on Lipide Accumulation.

No.	1	2	3
A) KNO_3 (mol/l)	0.01	0.0025	0.00025
B) Maximum density to be reached by normal cells* (corresponding to A)	2.0 g/l	0.5	0.05
C) Inoculum size (x B)	1/50	"	"
D) Final density	6.98 g/l	3.35	0.39
E) Culture length (Days)	15	10	6
F) D/B	3.49	6.70	7.90
G) Lipide %	58.5	79.0	—

* Contain 7% total nitrogen.

Light intensity: 4000 lux on both sides of the flask.—

grow and multiply remarkably, even in a completely nitrogen-free medium.

The difference of the final algal concentration, consequently that of the degree of nitrogen deficiency reached, corresponding to the different initial algal concentrations, is shown in Table II. The light intensity and the depth of culture are fixed in this experiment.

In culture No. 1, starting with the initial algal concentration of 4.58 g/l, the final algal concentration reached in ten days was only twice as high as the initial one, while in culture No. 7, starting with the initial concentration of one sixtyfourth of that of No. 1, namely 0.07 g/l, the final concentration reached after only four days was 11.4 times as high as the initial one. In the latter case, the lipide content was 84.5%. When culture No. 1, which had already reached maximum growth, was diluted with fresh nitrogen-free medium to one eighth and cultivation was continued, the growth curve after dilution showed quite a similar curve as culture No. 4 whose inoculum size was one eighth of No. 1, and the final concentration was 2.92 g/l, almost the same value as that of No. 4, 2.68 g/l.

This phenomenon is to be explained

by the fact that the amount of light energy absorbed by each cell in unit time is larger after dilution than that before dilution. It was ascertained that^{5, 8, 9)}, in the case of normal cells, the total increase of mass by photosynthesis per unit area is constant irrespective of the depth of culture and the algal concentration, if the incident light intensity is fixed and the light is completely absorbed by the suspension. Whereas if the light intensity is too weak, or if the algal density is too thick, it may happen that the net increase of mass does not correspond to the incident light energy, as the decrease of mass due to respiration becomes competitive to the increase due to photosynthesis⁹⁾.

The same phenomenon appears to occur in a greater extent in the case of nitrogen deficient cells, even when the light intensity is not so weak and the total algal mass per unit area is not so large. Considering the fact that further growth can be brought about by dilution even when the culture has reached maximum growth and does not show any more growth owing to the equilibrium described above, it seems that light energy

8) Y. Fujimoto, H. Iwamoto, A. Kato and K. Yamada This Bulletin, in print.

9) J. Myers, "Algal Culture", Carnegie Inst. Washington Pub. 1953 Chap. 4, p. 37.

Table II
Effect of the Initial Concentration of Algae on Nitrogen-free Growth.

No.	A Initial conc. g/l	B Final conc. g/l	C Culture length (Day)	D B/A	E Total N. %	F Lipide %
1	4.58	9.21	10	2.0	4.08	34.2
2	2.29	6.40	9	2.8	2.91	50.6
3	1.14	4.19	8	3.7	2.20	56.4
4	0.57	2.68	7	4.7	1.74	—
5	0.28	1.58	6	5.6	1.46	74.0
6	0.14	1.21	5	7.1	0.95	—
7	0.07	0.80	4	11.4	0.72	84.5

Light intensity: 4000 lux on both sides of the flask.

efficiency is very low in a nitrogen deficient cell compared with a normal cell. It was also mentioned by Milner³⁾ that a stronger light is required to obtained nitrogen deficient cells, while normal cells are able to grow even in a weak light.

In the case of culture No. 7, no further growth after dilution was observed. And so, it is believed that No. 7 had already reached the highest nitrogen deficiency which allowed no further growth. In this case the total nitrogen of the cells was 0.72%, in other words, the crude protein content was as small as 4.5%.

It is also conceivable that, if the depth of culture is varied, the final algal concentration will differ, even though both the initial concentration and the light intensity are not varied. This is true as indicated in Table III, the deeper culture showing the lower final concentration.

Further, it is quite reasonable to think that, if both the final concentration and the depth of culture are the same, the stronger the light intensity, the greater the growth rate is and the higher the final concentration would be reached. But the experiments showed, on the contrary,

Table III
Effect of the Depth of Culture on Nitrogen-free Growth.

No.	A Initial conc. g/l	B Final conc. g/l	C Depth. cm	D Culture length. day	E B/A
1	0.52	1.77	3.0	11	3.5
2	0.57	2.68	1.5	7	4.7

Light intensity: 4000 lux on one side of the flask.

Table IV
Effect of the Light Intensity on Nitrogen-free Growth. (I)

No.	Incident light. kilolux	Initial conc. g/l	Final conc. g/l	Culture length. day	Total N. %	Lipide. %
1	5	1.0	4.90*	18	—	—
2	10	1.0	5.05	18	1.56	63.7
3	30	1.0	4.27	18	1.81	55.0
4	50	1.0	3.97	18	2.02	50.6

* not maximum concentration.

Table V
Effect of the Light Intensity on Nitrogen-free Growth. (II)

No.	Incident light. kilolux	Initial conc. g/l	Final conc. g/l	Culture length. day	Total N. %	Lipide. %
1	10	1.75	6.00*	10	2.33	53.2
2	50	1.75	5.63*	10	2.49	51.0

* not maximum concentrations.

that not the stronger light but the weaker light results in the higher final concentration. It is seen, however, that the culture in the stronger light grows faster in the very initial stage than that in the weaker light. Tables IV and V, and Fig. 2 and 3 show these results.

As is seen in Fig. 3, the curves representing the potentiality in growth of the whole culture at every moment during cultivation, corresponding to the light intensities, 10 and 50 kilolux, intersect at

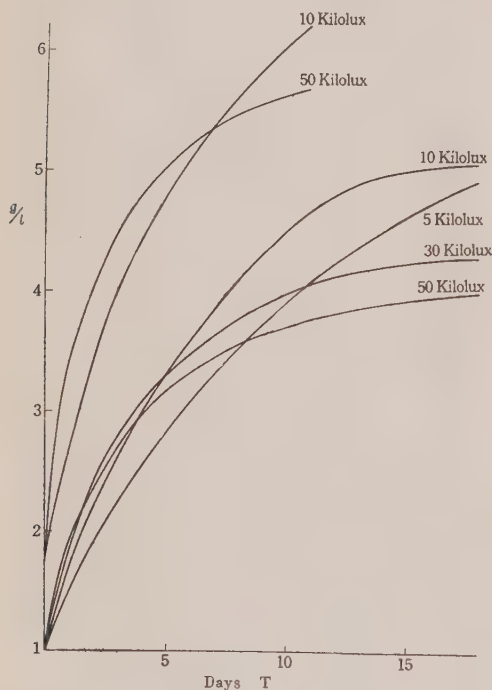


Fig. 2. Growth Curves in the Nitrogen Deficient Medium at Various Light Intensities.

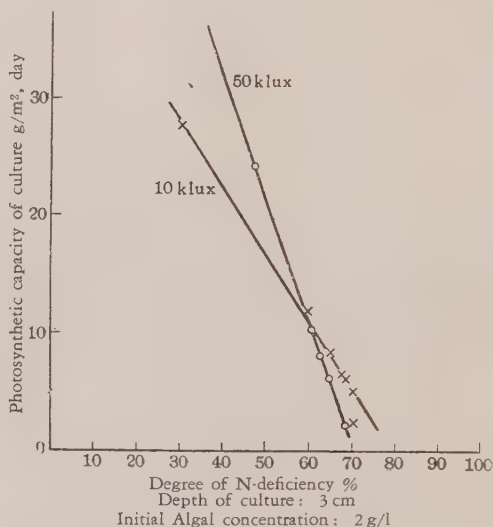


Fig. 3. Degradation of Photosynthetic Capacity of Nitrogen-free Culture.

the point of about 60% nitrogen deficiency. And the final concentration reached at 10 kilolux is higher than that at 50 kilolux as shown in Fig. 2 (upper curves). This is indicated more clearly by the lower curves which give a comparison of the light intensities 5, 10, 30 and 50 kilolux.

These results can be explained by the difference in the degree of chlorosis which occurs along with the progress of nitrogen deficiency of cells corresponding to the difference in the light intensity. The chlorophyll content of *Chlorella* cells grown in a nitrogen rich medium and in an algal suspension of very low algal density, is much smaller in the case of the light intensity higher than 5–10 kilolux than in the case of the lower light

intensity¹⁰⁾. This phenomenon, however, is not observed in a very dense culture. But in the case of nitrogen deficient cells, it is seen, even in a very dense culture. In Fig. 4, is shown the correlation between the degree of chlorosis and the degree of nitrogen deficiency during the growth at two light intensities, 10 and 50 kilolux, in the nitrogen free culture, having started with the algal density of 2 g/l. B represents 10 kilolux and C 50 kilolux. Supposed that chlorophyll is neither newly synthesized nor decomposed during the nitrogen-free growth, the curve is to be A. From Fig. 4 it is quite clear that the degree of chlorosis is much larger at 50 kilolux than at 10 kilolux and this difference grows larger with the progress of nitrogen deficiency. This is considered to be the reason why photosynthetic capacity becomes smaller at stronger light intensity that at a weaker one in the later stage of cultivation.

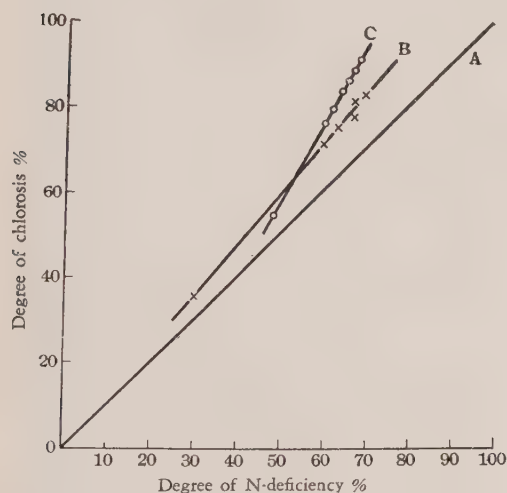


Fig. 4. Change of the Degree of Chlorosis during Nitrogen-free Cultivation.

Finally, the relation between the nitro-

10) Y. Fujimoto, H. Iwamoto and T. Ishii, This Bulletin, in print.

gen content and the lipid content of *Chlorella* cells is shown in Fig. 5, which indicates a very close relation between them.

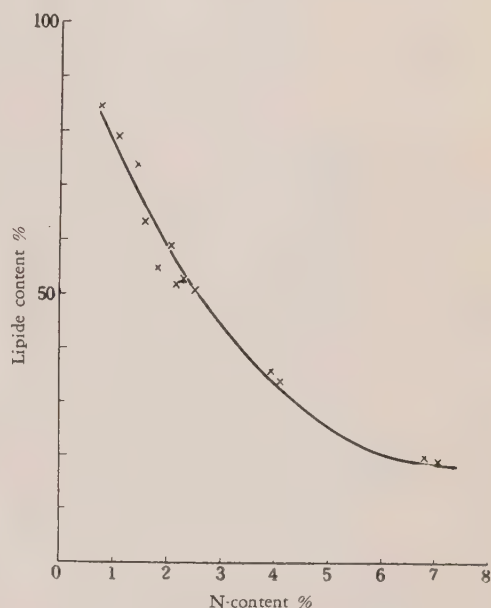


Fig. 5. Correlation between Nitrogen Content and Lipid Content of *Chlorella* cells.

Now, let us estimate the yield of fat on the basis of mass-production, calculating from Figs. 2 and 5. The yields of fat per 24 hours per square meter at various light intensities, and for various initial algal concentrations and date of harvest, are shown in Table VI. The time taken in producing the normal cells which is used in starting the nitrogen-free culture was taken into consideration in preparing this table. The percentage on the lower lines indicates the presumptive fat content on the harvest day.

It is interesting that there are no remarkable differences in the yields between the different conditions. The highest yield among them is 12 g/m²-day, which is obtained in the case of 50 kilolux, five days

Table VI
Yield of Lipide, g/m²; 24 hrs.

Incident light (kilolux)	5		10			50		
Culture length (day)	5	10	5	10	15	5	10	
Initial conc. (g/l)	1.0	5.2 (44%)	5.6 (59%)	8.4 (54%)	7.6 (64%)	6.1 (67%)	8.6 (51%)	5.9 (56%)
	1.75			8.8 (45%)	7.8 (53%)		12.0 (47%)	7.8 (51%)

Depth of culture: 3 cm.

Temperature: 25°C.

of culture length and 1.75 g/l of initial concentration.

The annual yield per acre is estimated as 4.9 ton on the basis of the daily yield, (8×10 24)g/m²-day., which is roughly considered as the average daily yield, under the conditions that each day has ten hours of illumination period of 10–50 kilolux of incident light intensity and the initial algal density is 1.0–2.0 g/l and the depth of culture layer is about 3 cm and the culture length is 5–10 days. This value might be much greater than the actual yield expected in the operation of outdoor culture, but it is believed to give a general standard. Moreover we should say that *Chlorella* gives an overwhelming high yield of fat compared with soybean oil or peanut oil, the former being about 100 kg/acre-year and the latter about 150 kg/acre-year.

Summary

In order to examine the possibility of mass-cultivation of *Chlorella* for the purpose of fat production, the cultural conditions for fat accumulation were studied,

Chlorella ellipsoidea being used. It was seen that *Chlorella* grew in the nitrogen-free medium, accumulating lipide in its cells, and that the accumulation degree was greatly affected by the initial algal concentration, the depth of the suspension and the incident light intensity. In order to obtain cells of a high lipide content, it was required that the initial density is low and the layer of the suspension thin. As for the light intensity, it was found that a stronger light has a stronger retarding effect on the nitrogen-free growth in its later stage than a weaker light over 5–10 kilolux. This is explained to be due to more severe chlorosis caused by the stronger light. The presumptive yield of fat by mass-culture was roughly estimated as 4.9 ton per acre per year under favourable conditions.

Acknowledgement

The authors are very happy to express their gratitude to Prof. H. Tamiya and Assistant Prof. K. Yamada for their cordial instruction and to Mr. Y. Fujimoto for his kind cooperation.

Fat Synthesis in Unicellular Algae.

Part II. Chemical Composition of Nitrogen-deficient *Chlorella* Cells.

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Received, October 18, 1955

In order to learn the relation between fat accumulation and carbohydrate metabolism in nitrogen-deficient *Chlorella* cells, the change of the fractions of carbohydrate during the progress of nitrogen-deficiency was studied in the first place. Some remarkable behaviour of certain fractions was observed. The change of chlorophyll and carotinoid content were also investigated.

In the previous paper¹⁾, it was reported that *Chlorella ellipsoidea* grew remarkably in the nitrogen-free medium, accumulating fat in its body, and that the total nitrogen compounds finally became less than 5% of the total dry weight. It was also ascertained that there is a close relation between the nitrogen content and lipid content of the cells. From the correlation curve between them and the growth curve, it is presumed that the accumulation substance in the early stage of nitrogen deficiency is not only lipid, while in the very advanced stage it is lipid alone. The substance other than lipid synthesized and accumulated in the early stage must be carbohydrate, because no new nitrogenous compounds are synthesized in the nitrogen-free medium.

It is an interesting problem in such an organism as *Chlorella* in which both photosynthesis and storage of the synthesized matters occur, whether fat is synthesized from carbohydrate pool, once synthesized and accumulated, or it is synthesized from some simpler substance without passing through carbohydrate.

Brown²⁾ presented the same question from the fact that he did not recognize any increase of carbohydrate in photosynthesis in *Scenedesmus*.

In this report, relating to this problem, the change of the fractions of carbohydrate during the progress of nitrogen deficiency is postulated.

Materials and Methods

The culture apparatus is shown in Fig. 1. The culture flask was of the same type as described in the previous paper¹⁾ but of a larger size, the capacity and the thickness being 2 liter and 3.5 cm respectively. Culture medium of 1.8 liter was put in each of four flasks which were set between the rows of fluorescent lamps of white light installed in a water bath. Each row consisted of five lumps. The light intensity at the surface of the flasks was about 4,000 lux.

The composition of the medium and the conditions of aeration and temperature were the same as those applied in the previous paper, except that urea was used as the nitrogen source in producing the normal cells.

In order to obtain the cells in various steps of nitrogen-deficiency, the following procedure of cultivation was carried out.

In the first place, *Chlorella* was grown in the complete medium. The normal cells thus obtained were collected by centrifugation and washed

1) H. Iwamoto, G. Yonekawa and T. Asai, This Bulletin, **19**, 240-246 (1955).

2) A. H. Brown, *Plant Physiol.*, **23**, 331-337 (1948).

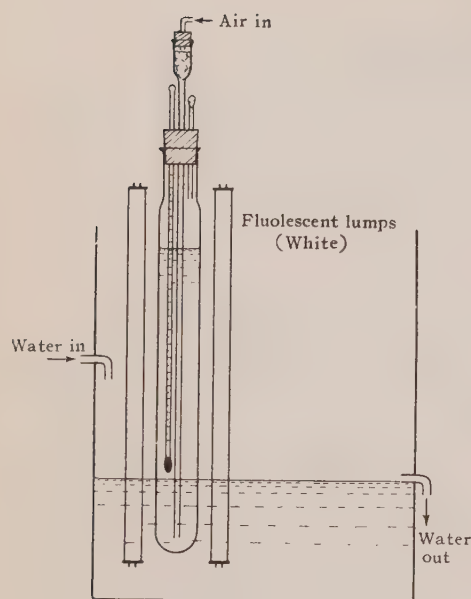


Fig. 1. Culture Apparatus.

twice by distilled water and resuspended in the nitrogen-free medium at the cell concentration of about 1g/l. Aeration and illumination were continued until the cell concentration became twice as large as the initial one. About one half of the cell suspension was then drawn out and the cells were centrifuged, washed twice and used as the sample of the first step of nitrogen deficiency. The rest of the suspension was diluted with the same volume of fresh nitrogen-free medium and cultivation was continued until the cell concentration increased about twofold again. The same procedure was repeated three times and the cells were collected at each time, and thus three kinds of nitrogen-deficient cells were obtained.

This procedure of dilution in sampling is necessary to obtain the cells of high nitrogen deficiency, because nitrogen deficient cells grow very slowly at a high population density and extremely nitrogen-deficient cells are produced only at a low population density, as already reported¹⁾.

The nitrogen content of the samples analyzed, one series of samples out of four series thus obtained with four flasks, is shown in Table I.

The fresh cells harvested as the samples were

Table I
Nitrogen Content of the Samples.

Sample No.	0	1	2	3
N %	7.11	3.89	2.44	1.59

dried on the tonplates in air, collected and stored in a vacuum desiccator. The dried samples of 3-4% moisture content were subjected to extraction and analysis.

In the analysis of pigments, fresh cells instead of dried cells were used as the samples, because chlorophyll in dried cells is very easy to fade especially in the light.

The methods of moisture and lipid measurement were the same as those described in the previous paper. Reducing sugar determination was pursued by modified Somogyi method. Colorimetry was applied to ketose determination after Roe³⁾, and Brown's dichromatic colorimetry⁴⁾ was applied to pentose determination. Extraction and determination of pigments were accomplished after Haskin⁵⁾ and those of carbohydrate were accomplished by a procedure similar to that employed by Smith⁶⁾ and Brown²⁾. The flow sheet of the extraction and separation processes is shown in Table II.

A dried sample 0.8 g was extracted with 20ml. of 80% ethanol five times for 20 min. each on a water bath under reflux. The alcoholic extract was evaporated to a small amount on the water bath. Water was added and the solution was evaporated until the odor of alcohol had disappeared. This procedure was repeated three times. The concentrated solution was added with 1ml. of 14% lead acetate solution, filtered, added with 2 ml. of saturated sodium oxalate, and filtered again.

The filtrate was made to volume 100 ml. Ten ml. out of 100 ml. was subjected to free reducing sugar determination, 40 ml. was added with HCl to 1% and hydrolyzed in the boiling water bath for 20 min., and 20 ml. was hydrolyzed with 1N HCl for 4 hrs. at 100°C. The hydrolyzates were made to volume 50 ml. after neutralization with NaOH. Sucrose was determined with the former hydrolyzate, and ex-

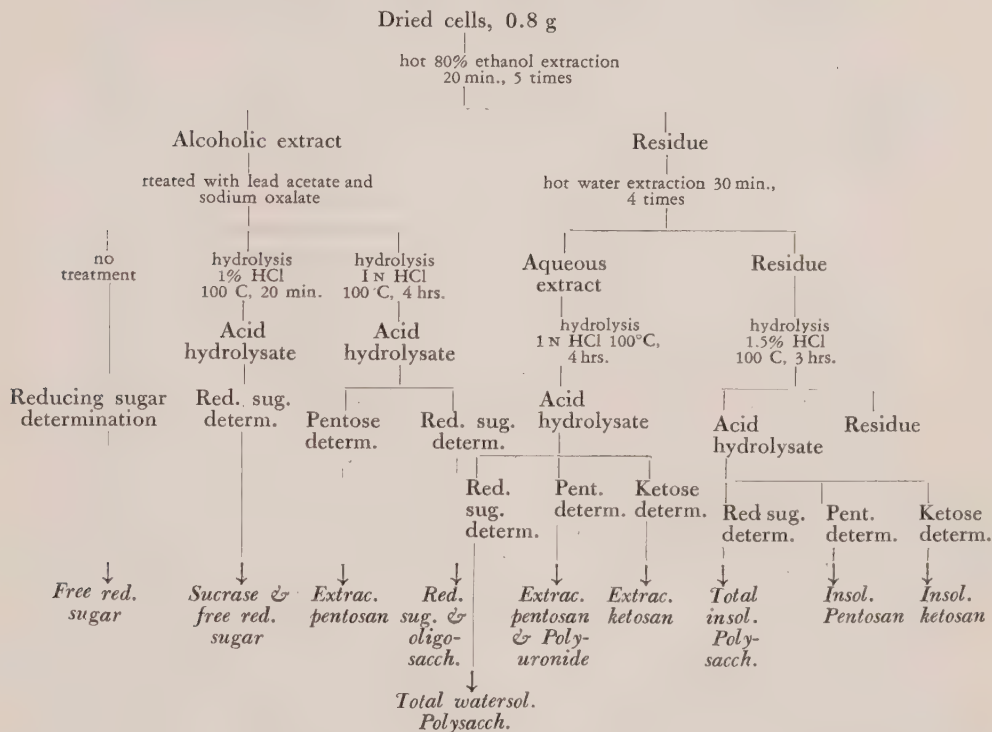
3) J. H. Roe, *J. Biol. Chem.*, **107**, 15-22 (1934).

4) A. H. Brown, *Arch. Biochem.*, **11**, 269-278 (1946).

5) H. H. Haskin, *J. Biol. Chem.*, **144**, 149-160 (1942).

6) J. H. C. Smith, *Plant Physiol.*, **18**, 207-223 (1943).

Table II
Flow Sheet of Extraction and Separation of Carbohydrate.



tractable pentosan and oligosaccharide with the latter.

The residue of alcoholic extraction was extracted with 20 ml. hot water for 30 min. three times. The extract was hydrolyzed with 1N HCl at 100°C for 4 hrs., concentrated to 30 ml. and neutralized with 1N NaOH and made to volume 50 ml. This hydrolysate was divided into three parts and subjected to reducing sugar determination, pentose determination and ketose determination. These determinations gave total water-soluble polysaccharide, extractable pentosan plus polyuronide and extractable ketosan respectively.

The residue of aqueous extraction was hydrolyzed with 20 ml. of 1.5% HCl for 3 hrs., filtered, and washed with 20 ml. hot water three times and 15 ml. hot water once. The filtrate was neutralized and made to volume 100 ml. The same determinations as those on the aqueous extract were made on this filtrate, giving total

insoluble polysaccharide, insoluble pentosan and insoluble ketosan respectively.

Results

The result of analysis on the hot alcohol extract is shown in Table III. The amount of free reducing sugar was 2.9% in the normal cell and increased with the progress of nitrogen deficiency in the cell, and this reached 11.4%, when the nitrogen content became about one fourth of that of normal cell. The amount of sucrose, which was 11.2% in the normal cell, on the contrary, decreased very quickly and disappeared at the nitrogen level of one-third of the normal cell. It is quite presumable²⁾ that the normal cell has a large amount of sucrose. But it is

Table III
Analysis of Hot 80% Alcohol Extract. (per cent to the total dry matter)

Sample No.	0	1	2	3
Free reducing sugar (as glucose)	2.9	3.1	7.9	11.3
Sucrose	11.2	4.8	0	0
Extractable pentosan	trace	"	"	"
Oligosaccharide (as glucose)	3.1	2.7	1.4	0

Table IV
Analysis of Hot Water Extract.

Sample No.	0	1	2	3
Total water soluble polysaccharide (as glucose)	0.9	1.7	2.1	2.0
Extractable pentosan and polyuronide	trace	"	"	"
Extractable ketosan	trace	"	"	"

a very remarkable phenomenon that it decreases so rapidly, along with the increase of free reducing sugar. Oligosaccharide other than sucrose had the same trend as sucrose and disappeared in sample No. 3.

The analysis on the hot water extract is shown in Table IV. In this fraction, water soluble polysaccharide showed a gradual increase with the progress of nitrogen deficiency.

Pentose and ketose determination in above two extracts showed no noticeable

amounts of them in any stage of nitrogen deficiency.

The result on the hot 1.5% HCl extract is shown in Table V. Insoluble polysaccharide made a big fraction. It showed a gradual decrease after increasing in the early stage. A small amount of insoluble pentosan was found in any stage of deficiency, showing gradual decrease.

The changes of total carbohydrate, crude protein and crude lipide are shown in Table VI. Crude protein was estimated

Table V
Analysis of Hot 1.5% HCl Hydrolysate.

Sample No.	0	1	2	3
Total insoluble polysaccharide	9.6	16.6	15.7	9.2
Insoluble pentosan	2.2	2.0	1.4	1.4
Insoluble ketosan	trace	"	"	"

Table VI
Changes of Protein, Carbohydrate and Lipide Content with the Progress of Nitrogen Deficiency. (%)

Sample No.	0	1	2	3
Crude protein	44.5	24.3	15.3	9.9
Carbohydrate	29.9	30.9	28.5	23.9
Crude lipide	18.5	36.1	50.2	63.7

Table VII
Changes of the Amount of Each Component of Cells in a Whole Culture during
Growth in N-free Medium (Relative values).

Sample No.	0	1	2	3
Total drymatter	1	1.83	2.91	4.47
Total protein	1	1	1	1
Total carbohydrate	1	1.87	2.77	3.58
Total lipide	1	3.58	7.91	15.4

Table VIII
Changes of Pigments with the Nitrogen Deficiency.

	Days of cultivation	0	1	2	3	4	5	6	7
	N-content*	100	65	51	44		36		33
10	Chlorophyll %	5.50	3.50	2.72	2.21	1.52	1.37		1.11
kilolux	Carotenoid %	0.480		0.141	0.125				0.050
	Chl./Car.	11.5			17.7				22.5
	N-content*	100	51	44	39		35		33
50	Chlorophyll %	5.50	2.51	1.85	1.34	1.12	0.90	0.81	0.64
kilolux	Carotenoid %	0.480		1.109					0.062
	Chl./Car.	11.5		12.6					10.6

* relative values.

by multiplying the nitrogen content by 6.25. It is observed that the carbohydrate content did not change conspicuously, before the protein content decreased to about one-third of the normal cell and crude lipide increased to about 50% approximately three times as much as that of the normal cell, and that it decreased gradually with further nitrogen deficiency.

It was found previously that lipide accumulated to 84.5%, when crude protein became 4.5%. In that case, the amount of total carbohydrate must have been about 5% or so. And so, free reducing sugar and insoluble polysaccharide must decrease to a very small amount with further progress in nitrogen deficiency.

Table VII shows the relative amounts of total dry matter, total crude protein, total carbohydrate and total crude lipide, contained in a batch of culture, which, except total protein, vary with nitrogen deficiency

in each individual cell.

The initial amount of each of these materials was made unit in order to see clearly the feature of synthesis of each material. From the table, it is clear that more lipide is synthesized and accumulated in the earlier stage of nitrogen deficiency by about the same amount as the protein which would be synthesized when grown in the complete medium, but in the advanced stage, synthesis of carbohydrate is also gradually replaced by synthesis of lipide.

Von Witsch and Harder⁷⁾ described on the relation between fat and the rest materials that fat storage was not produced at the expense of the rest of organic matter contained in the cells. But this is true only in the earlier stage of fat storage, up to about 50% of total

7) von Witsch and R. Harder, *Garnegie Inst. Wash. Pub.*, 154-165 (1953).

dry weight, and after that it occurs not only that carbohydrate synthesis is gradually replaced by fat synthesis but that in the very advanced stage the carbohydrate once accumulated is converted to fat.

As for sucrose, its synthesis ceases and hydrolysis occurs in the very early stage of nitrogen deficiency. The accumulation of free reducing sugar is considered partly due to the hydrolysis of sucrose, and the accumulation of soluble and insoluble polysaccharide might be due to the accumulation of free reducing sugar.

As already reported¹⁾, a very remarkable change of pigment content occurs in nitrogen deficient cells. The changes of chlorophyll and carotenoid content and the chlorophyll/carotenoid ratio with the progress of nitrogen deficiency during nitrogen-free cultivation at two different light intensities are shown in Table VIII.

Chlorophyll decreased from 5.5% to 1.11% at 10 kilolux and from 5.5% to 0.64% at 50 kilolux, while, carotenoid decreased from 0.48% to 0.05% at 10 kilolux and to 0.062% at 50 kilolux, when the nitrogen content was reduced to one-third of the normal cells. Accordingly the chlorophyll/carotenoid ratio changed from 11.5 to 22.2 at 10 kilolux and did not change remarkably at 50 kilolux. The greater part of carotenoid was xanthophyll. The amount of carotene in the normal cells was 0.118% and it disappeared prior to the third day in the cultivation.

It was mentioned in the previous paper that chlorosis occurred in nitrogen deficient cells in a more intense degree at

the higher light intensity.

As for carotenoid, it seems to show no marked difference in their decreasing velocity at different light intensities. Generally speaking, carotenoid seems to decrease faster than chlorophyll.

Summary

Changes of carbohydrate fractions and chlorophyll and carotenoid content in the cells during the nitrogen-free cultivation were studied. It was found that free reducing sugar increased and sucrose decreased remarkably with the progress of nitrogen deficiency in the cells. Soluble and insoluble polysaccharides were also found to increase in the earlier stage. It is considered that fat is not synthesized at the expense of carbohydrate in the early stage of fat accumulation, up to 50%, but that, in the later stage, not only the synthesis of carbohydrate is reduced, but the accumulated carbohydrate, the greater part of which is free reducing sugar, is converted to fat.

The chlorophyll content diminishes faster than the nitrogen content, especially at high light intensity. Carotenoid decreases as fast as or faster than chlorophyll, and carotene disappears in the very early stage of N-deficiency.

Acknowledgement

The authors are greatly indebted to Prof. T. Asai, Prof. H. Tamiya and Assistant Prof. K. Yamada for the accomplishment of this work. And they also wish to thank Mr. Y. Fujimoto and Mr. G. Yonekawa for their kind help.

The Influence of Magnesium Deficiency on the Growth of *Chlorella*.

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The growth of *Chlorella* in the magnesium deficient medium was postulated. It was found that magnesium deficiency has greater influence upon multiplication of cells than upon synthesis of cell materials. And the size of cells becomes larger in the magnesium deficient medium. Chlorophyll formation is completely suppressed by the deficiency. But it seems that growth is affected by magnesium deficiency itself rather than by chlorophyll deficiency caused by magnesium deficiency.

In the batchwise cultivation of unicellular green algae, *Chlorella ellipsoidea*, it has often been seen that the growth rate of cells slowed down in the later stages of culture, showing inactive multiplication, until the increase of cells ceases and the cell digestion occurs. This phenomenon was considered to be due to some unsuitable environmental condition for the cell growth, especially the deficiency of nutrients in the culture solution.

On *Chlorella*^{1, 2, 3)} and a few other green algae^{4, 5)}, it has been noticed that the photosynthetic activity of cells is greatly correlated with the chlorophyll content and that the synthesis of chlorophyll, which is a magnesium porphyrin, is affected by the magnesium content of the culture solution. Recent findings^{6, 7)} indicate that magnesium is required also

for the catalase synthesis, and that the process of multiplication may be an independent process from the synthesis of cell material.

In the present study, changes in the cell volume, cell counts and chlorophyll content, during the course of growth were followed, and the level of magnesium requirement was investigated.

Experimental Methods

The strain used was *Chlorella ellipsoidea*, and the full nutrient solution contained the following components per liter: KNO₃, 5.0 g; Mg SO₄·7H₂O, 2.5 g; KH₂PO₄, 1.25 g; FeSO₄·7H₂O, 0.003 g; the microelements B, Mn, Zn, Cu and Mo in the same amounts as used by Arnon⁸⁾. The pH was 5.1 to 5.8. In the magnesium-free media, equivalent amounts of K₂SO₄ were substituted for MgSO₄ for SO₄²⁻. An oblong and flat flask with the inner thickness of 2.8 cm and a total capacity of 600 ml., which was originally used by Tamiya et al⁹⁾ was filled with 350 ml. of culture medium. The cultures were continuously aerated with air containing 5 percent CO₂ at a rate of 350 ml. per minute which also served for homogenizing the suspen-

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- 6) B. J. Finkle and D. Appleman, *Plant Physiol.*, **28**, 652-663, (1953).
- 7) B. J. Finkle and D. Appleman, *ibid.*, **28**: 664-673, (1953).

- 8) D. I. Arnon, *Am. J. Bot.*, **25**, 322-325, (1938).
- 9) H. Tamiya et al., *Carnegie Inst. Wash. Pub.*, **600**, 204-232, (1953).

sion.

The culture flasks were illuminated on one side by a reflector flood lamp placed outside the thermostat, which was maintained at 25°C. The light intensity at the surface of the culture flask was about 15 kilolux.

To study the effect of magnesium deficiency, some cultures were dealt with the magnesium-free media. The culture solution was refreshed once a day so as to keep the composition of the nutrient constant throughout the experiments. As will be described¹⁰⁾, the increase of cells per unit area varies with its density in low population density, so that the cultivation was done in the linear phase in which the yield per unit area in the complete nutrient solution was almost constant at the fixed light intensity.

The cell growth was followed by measuring the volume of packed cells¹⁰⁾, the cell counts in a standard haemocytometer, and the dry weight determined after drying at 100°C for 24 hrs. The calculation of algal size was made, by dividing the volume of packed cells by the number of cells. Chlorophyll was extracted from the cells by hot methanol as postulated by Fleischer¹⁾ and the determination of it was done after Haskin's method¹¹⁾.

Experimental Results

An active culture of about 4 ml./l of cell concentration was divided into two parts. The cells in one part were subjected to cultivation in the full nutrient medium and the cells in the other part were centrifuged, washed and resuspended in the magnesium-free medium. The course of growth, followed with the cell population and volume of packed cells, is shown in Fig. 1, in which the circles denote the data in the full nutrient medium and the crosses are the data in the magnesium deficient medium. The increase of cells in the former showed a typical linear growth and was accompanied with the proportional increase of population. On the other hand, in the magnesium deficient condition, the volume of packed cells increased 3.5 times after 8 to 10 days, though growth rate was far lower than in the case of the full nutrient medium, but the

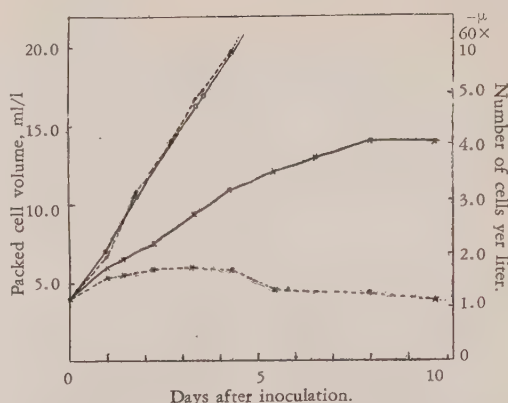


Fig. 1. Growth in the Linear Phase with and without Magnesium.

population after 3–4 days, was only 1.5 times as high as the initial. And the diminution in cell counts was apparent in the later stage of culture.

The difference between the packed cell volume and population was induced by the change in cell size during the course of growth. That the cell size changes with the change of culture conditions and with growth age, has been already noticed^{12,13,14,15)} and also the variability due to magnesium deficiency was pointed out. During the active growth in the full nutrient medium, changes in cell size have been scarcely seen and the average size and radius were about $35\mu^3$ and 2.0μ respectively, as shown by the circles in Fig. 2. On the contrary, in the magnesium-free culture, the increase in cell size began at once and cell enlargement continued until the cell volume became 3.5 times as large, giving a change in the radius by 1.5 times, as shown by the crosses in Fig. 2. The dry weight was proportional to the packed cell volume even after the cessation of cell multiplication, so that an increase in cell size was considered to be based on the increase in cell material and not on the swelling of the cells by water uptake⁷⁾.

It is also noticeable that the growth activity of cells is affected by the change of the chlorophyll

10) Y. Fujimoto et al. This Bulletin, in print.

11) H. H. Haskin, *J. Biol. Chem.*, **144**, 149–160, (1942).

12) W. H. Pearsall and L. Loose, *Proc. Roy. Soc.*, B **121**, 45–501, (1936).

13) H. Tamiya et al, *Carnegie Inst. Wash. Pub.* **600**, 76–84, (1953).

14) H. W. Milner, *ibid.* 108–113, (1953).

15) E. A. Davis and J. Dedrick, *ibid.* 138–143, (1953).

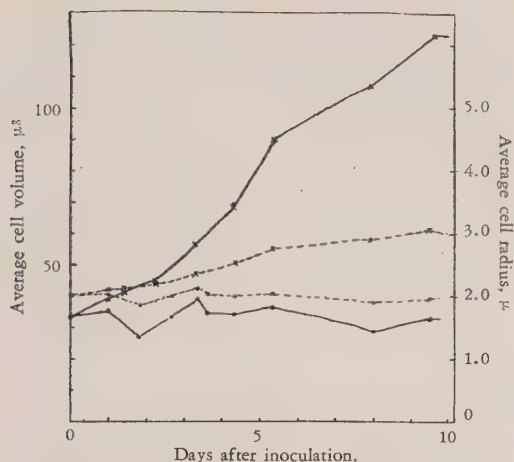


Fig. 2. Changes of Cell Size during Cultivation.

content due to magnesium deficiency^{1,2,3}), and that the level of magnesium in the original medium determines the degree of chlorophyll development⁶). The changes in chlorophyll content per unit volume of cell substance is scarcely seen in the case of the full nutrient medium, and the rate of increase of chlorophyll per unit volume of culture fluid is similar to the

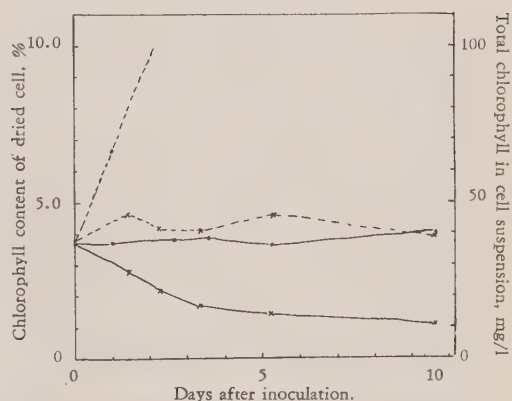


Fig. 3. Changes of Chlorophyll during Cultivation.

rate of increase of population as shown by the circles in Fig. 3.

In the magnesium deficient medium, chlorophyll content per unit volume of cell substance gradually decreased, but that per unit volume of culture fluid was almost invariable as the probable result of cessation of chlorophyll formation. This was also clear from the fact that the addition of magnesium to such a condition caused a vigorous recovery—the chlorophyll content increas-

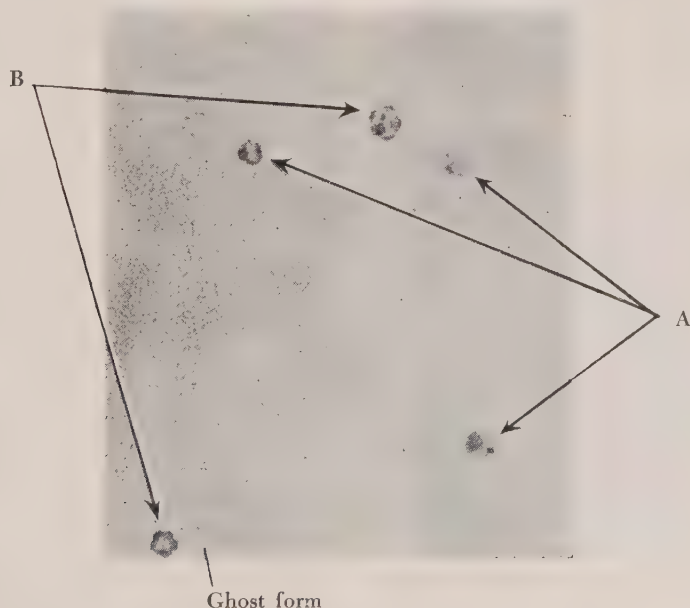


Fig. 4. Stage of cell digestion. $\times 510$

ed at once, and the trend of change in cell size was reversed and cell growth was remarkably activated until the linear growth was seen.

When the cultivation was further continued in the magnesium-free medium, the digestion of cells was seen, being followed by the decrease of cell count in the culture solution. The digestion occurred ordinarily in the large and non-dividing cells which were apparently thick-walled. These cells are multicellular units, but the division of cell material is imperfect, or sometimes the hindrance of separation was seen, accompanied with the division of cell material as shown in Fig. 4-A.

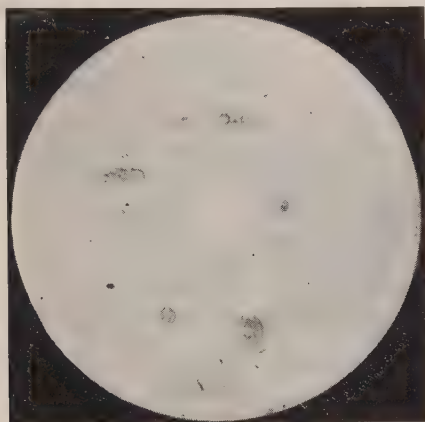


Fig. 5. Appearance of cell digestion.
× 750

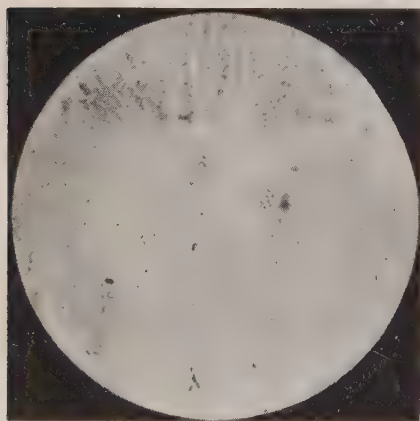


Fig. 6. The ghost form of cell wall, and cell material pushed out.
× 750

In the early stage of digestion, the detachment of cell material from the cell wall was found as shown in Fig. 4-B, and the cell materials were gradually crowded out of the cell wall with an oval form as shown in Fig. 5. These observations at hand were not adequate to confirm the cause of dehiscence of cell wall and separation of cell materials from cell wall, but this may be a problem of physiological interest.

After the complete separation the cell wall remained as a ghost form, while the amorphous cell materials were broken up in the culture solution as shown in Fig. 6.

Discussion

It is probable that the process of cell division and of accumulation of cell material are limited by different metabolic factors requiring different levels of magnesium concentration.

In the full nutrient medium, the cell division and synthesis of cell material are almost proportional to each other, while the rate of cell division is smaller than that of formation of cell material in the magnesium deficient medium. The tendency that the synthesized material was used largely for increasing cell size rather than for producing new cells became conspicuous at the later stage of culture development, and yet the synthesis of cell material continued even after the cell division ceased.

These facts clearly indicate that the synthesis of cell material can take place at a lower level of magnesium in the cell than that required for cell division, and it appears that, when the accumulation of cell material stopped, the digestion occurred with dehiscence of thick cell wall in the step of its multicellular units.

In the full nutrient medium, the production of chlorophyll was followed by synthesis of other cell constituents and the chlorophyll content in cells was usually constant in the stationary culture

condition. But the chlorophyll synthesis in magnesium-free condition was almost negligible and the decline of activity of photosynthesis was remarkable even at the earlier stage of cultivation. Noticeable is the fact that the production of cell materials continued after the synthesis of chlorophyll ceased. These may suggest that the synthesis of chlorophyll requires a higher level of magnesium concentration than cell division or production of other cell materials.

It is of interest to compare the magnesium deficiency with the nitrogen deficient condition. Even in the nitrogen-free but full magnesium content medium, the growth and the multiplication were almost the same in their activity as in the full nutrient medium for a fairly long time during the earlier stage of cell growth, although fat body was produced owing to the nitrogen deficiency.

As already reported¹⁶⁾, the cells of low chlorophyll content are produced at an active rate of synthesis of cell material and multiplication, and the activity is almost proportional to the chlorophyll content. But in the magnesium deficient medium, the activity is not proportional to the chlorophyll content. The activity of synthesis of cell material drops lower and that of multiplication drops much lower than in the case of nitrogen defi-

ciency, even if the chlorophyll content is the same in both cases.

Summary

Magnesium as a controlling factor in the multiplication and the formation of cell material or chlorophyll has been investigated at about 15 kilolux, being contrasted with the full nutrient or the nitrogen-free condition. In the full nutrient culture, the relative relation of these processes proceeded in a rate almost constant and the cells of about 3.5% chlorophyll content were produced in the same size at the same linear growth rate. The cell growth was continued even without taking magnesium, giving the cell size more than 3.5 times larger in volume than the normal cell, and the cell digestion occurred in the later stage of culture. From the sequence in which the activity of the respective processes decreased, the level of magnesium requirement was suggested. It is probable that the formation of chlorophyll takes place at the highest level of magnesium in the cell, and the synthesis of other cell materials occurs at lower level than the cell division.

Acknowledgement

The authors are indebted to Prof. T. Asai, Prof. H. Tamiya and Assist. Prof. K. Yamada for their instruction and to Mr. H. Iwamoto for his kind cooperation.

¹⁶⁾ H. Iwamoto, G. Yonekawa and T. Asai, *This Bulletin*, **19**, 240-246 (1955).

Islanditoxin, a Toxic Metabolite Produced by *Penicillium islandicum* Sopp.

Part I.

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Received October 21, 1955

Islanditoxin^{1, 2)} has been isolated from the cultured broth of *Penicillium islandicum*, and $C_{25}H_{35}O_8N_5Cl_2$ was proposed as its molecular formula. The presence of the peptide linkage in islanditoxin was ascertained and the general properties were described.

It was known that there were three kinds of poisonous yellowsis rice, i.e. the citrinum yellowsis rice³⁾ caused by *Penicillium citrinum*, the so-called yellowsis rice⁴⁾ caused by *Penicillium toxicarium* and the islandia yellowsis rice⁵⁾ caused by *Penicillium islandicum*. The poisonous substances of the former two were found to be citrinin⁶⁾ and $C_{27}H_{36}O_7$ ⁷⁾. Whereas, that of the latter still remains undetermined, and it has become an important problem to investigate the toxic substance produced by *Penicillium islandicum*, especially in Japan.

B. H. Howord and H. Raistrick isolated six colouring matters from the cultured mycellium, namely islandicin⁸⁾, iridoskyrin⁹⁾, rubroskyrin⁹⁾, erythroskyrin⁹⁾, flavo-

skyrin¹⁰⁾ and skyrin¹⁰⁾, but no appreciable toxicity was found in them.

The three toxic substances have been reported already as the toxic metabolite produced by *Penicillium islandicum*. Islanditoxin^{1, 2)}, isolated by Y. Sumiki and the author, had a toxicity of minimum lethal dose 30 γ /10 g in mice given subcutaneously. Pigment X¹¹⁾, isolated from the cultured mycellium by T. Tatsuno, M. Tsukioka and et. al., showed the toxicity of 1.0 mg/10 g in mice given subcutaneously. Malonic acid was found by T. Yamamoto^{12, 13)} from the cultured broth with the toxicity of LD₅₀ 1.4 mg/10 g in mice. In the subcutaneous injection islanditoxin showed the strongest toxicity of the above three.

The present paper deals with the isolation, molecular formula and general properties of islanditoxin.

Penicillium islandicum was inoculated for 14 days at 28° on a Czapek-Dox medium containing 5% of sucrose as a carbon source. The isolation and purification

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2) S. Marumo, K. Miyao and A. Matsuyama *J. Agr. Chem. Soc. Jap.* **29**, 913, (1955).

3) H. Tsunoda *Report of the Food Research Institute* No. **8**, 77 (1953) No. **9** 169 (1954).

4) K. Uraguchi *Jap. J. of medical progress* **34**, No. 3 (1942)-**42**, No. 10 (1955).

5) H. Tsunoda: *Report of the Food Research Institute* No. **8**, 41 (1950).

6) Johnson, Robertson and Whalley: *J. Chem. Soc.* 1563 (1949), 2471 (1950).

7) Y. Hirata: *J. Chem. Soc. of Jap.* **68**, 63, 104 (1940).

8) B. H. Howord and H. Raistrick: *Biochem. J.* **44**, 227 (1947).

9) *ibid.* **57**, 212 (1954)

10) *ibid.* **56**, 56 (1954).

11) T. Tatsuno, M. Tsukioka, Y. Sakai, K. Suzuki and J. Asami: Monthly Meeting of Pharm. Soc. of Japan on July, 9 (1955).

12) T. Yamamoto: *J. Pharm. Soc. of Jap.* **75**, 512 (1955).

13) T. Yamamoto: *ibid.* **75**, 761 (1955).

were carried on with help of tests by the subcutaneous injection method in mice on each step. Islanditoxin, obtained from the cultured broth as a colourless crystalline of needles, melted with decomposition at 250–251°. The molecular formula of islanditoxin was in agreement with $C_{25}H_{33}O_8N_5Cl_2$, based on the elementary analysis and molecular weight determination by the cryoscopic method with phenol as a solvent. Methoxyl and N-methyl groups were not found in islanditoxin. $[\alpha]_D^{21} -47.7^\circ$ (c, 1.99 in acetic acid).

The ultraviolet absorption spectrum, measured on Beckman Spectrophotometer in aqueous solution, showed the absorption maximum at 257 $m\mu$ (ϵ 290), which lead us to the assumption that one benzene ring existed in one molecule of islanditoxin.

The infrared absorption spectrum, measured on Perkin Ermer double beam instrument as Nujol mull, showed a series of maximum bands 3450 (bonded OH) 3270 (bonded NH) 1650 (amide CO) 1530 ($>NH$) and 700 cm^{-1} (C–Cl). Some of those bands indicated the peptide linkage in islanditoxin, which was ascertained by the colour test of the hydroxamic acid reaction and finally to isolate the three kinds of amino acids from the acid hydrolysate of islanditoxin in the form of crystals.

Islanditoxin was readily soluble in acetone, butanol and phenol, soluble in methanol, ethanol, cyclohexanol, dioxane and water, slightly or not soluble in chloroform, ethylacetate, ether, benzene, petroleum ether and ligroin. Islanditoxin was more readily soluble in aqueous acidic and basic solutions than in neutral, this presuming that the islanditoxin was an amphoteric substance, but on observation of the infrared absorption spectrum the presence of a free carboxyl and amino

radical was not found.

Islanditoxin was stable in acidic and neutral solutions, but in basic quite labile. When the N/100-ammonia solution of islanditoxin was allowed to stand overnight at room temperature, the toxicity was completely lost, following the elimination of chlorine as its ionic form.

In the histopathological examination, the degeneration of liver cells and strasis of circulating blood were observed in the liver of mice injected with islanditoxin.

Experimental

Organism and cultural condition.

Penicillium islandicum, obtained through the courtesy of Dr. Tsunoda (Food Agency, Ministry of Agriculture and Forestry.), was used for the production of islanditoxin throughout this work.

The optimum condition producing islanditoxin in the stationary culture was examined. As the result, the constant yield of islanditoxin was obtained inoculating and incubating on Czapek-Dox medium (sucrose 50 g, K_2HPO_4 1g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $NaNO_3$ 2 g, KCl 0.5 g, $FeSO_4$ 0.01g, distilled water 1 l.).¹ The production of islanditoxin was not appreciably affected by the incubating temperature in the range of 25–33°.

A comparison of cultural condition between the surface and submerged cultures was drawn. The surface culture for 14 days at 28° (4 l. of medium in 8 l. of a orbicular aluminium vessel) gave 2–3mg of islanditoxin per 1 l. of cultured broth. On the shake-culture for 6 days at the same temperature (200 ml. of medium in 500 ml. of a shaking flask) about 1.5 mg of islanditoxin was recovered. The submerged culture using a glass jar fermentor (7 l. of medium in 10 l. of a glass fermentor, 1 l. of air/l. of medium/minute) gave 1.5–1.0 mg. But in the tank culture, islanditoxin could not be found in the cultured broth on account of an unknown reason. From results of this examination, the surface culture seemed to be more suitable for production of islanditoxin. The spore suspension of *Penicillium islandicum* was inoculated to 4 l. of medium in an orbicular aluminium vessel of 8 l. volume and incubated for

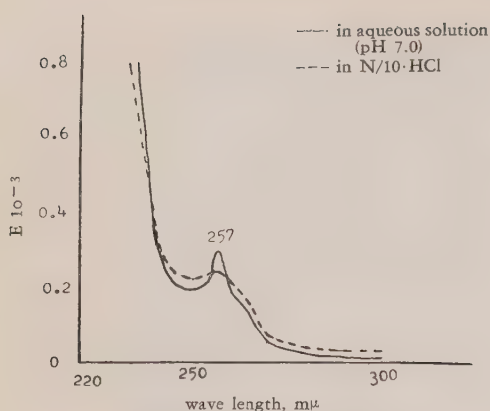


Fig. 1. Ultraviolet Absorption Spectrum of Islanditoxin.

14 days at 28°. The cultured broth gave a dark red colour and the final pH was 3.4.

The Test of Toxicity.

In each step of isolation and purification procedures, ten mice, of 15–20 g body weight, were selected. The samples injected in mice were dissolved in water and adjusted to pH 7.0 with hydrochloric acid or sodium hydroxide. In the case of water insoluble acidic substances, they were dissolved in an alkali solution and immediately back titrated to pH 7.0. (the basic substance was not found in cultured broth). The toxicity of the samples was determined observing the behavior of mice, lethal dose, lethal times and macroscopic anatomy after subcutaneous injection.

Isolation of islanditoxin.

The cultured filtrate, separated from mycellium,

was concentrated to 1/20 volume *in vacuo* at 60–70° and an 8 times volume of acetone was added to this solution. After separating the viscous precipitates, the aqueous acetone solution was concentrated until no acetone remained (pH 2.2). After a few hours, the precipitated acidic substances were filtered. The filtrate was adjusted to pH 7.0, and then extracted three times with 1/3 volume of butanol. The butanol extracts combined were subjected to eight plates counter-current distribution using butanol and M/5 Sörensen phosphate buffer (pH 7.0) as a solvent system. The contents of tubes 7 and 8 were combined, evaporated to dryness and crystallized from hot methanol. The crude crystal thus obtained was recrystallized from hot methanol and a mixture of ethanol and ligroin several times. To rise the m.p. up to 250–251°, it was necessary to use the above two kinds of solvents successively. About 2.5 mg of crude crystal was obtained from 1 l. of the cultured broth.

Anal. Found: C, 49.81; H, 5.62; N, 11.85; Cl, 11.58. Calcd. for $C_{25}H_{33}O_8N_5Cl_2$: C, 49.83; H, 5.48; N, 11.62; Cl, 11.79.

Molecular weight.

Determination of molecular weight was executed by the cryoscopic method, according to the Wilson and Heron procedure¹⁴. The value obtained was 564 (Calcd. 602 for $C_{25}H_{33}O_8N_5Cl_2$). Phenol was purified by adding 0.1% of aluminium and 0.05% of sodium bicarbonate. The mixture was distilled at atmospheric pressure until the azeotropes were removed, and then distilled at

14) Wilson and Heron: *J. Chem. Soc. Ind. (London)* **60**, 168 (1941).

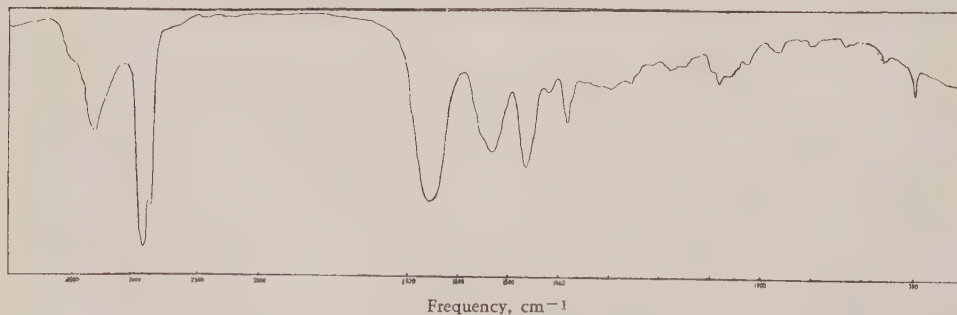


Fig. 2. The Infrared Absorption Spectrum of Islanditoxin as Nujol Mull.

25mm until 20ml. of black residue remained in the distillation flask.

Acknowledgement

The author wishes to express his sincere thanks to Prof. Y. Sumiki for his guidance throughout this work, and also to Mr. K. Ishida, Pathology Laboratory, Veterinary Science in our Faculty, for his histopa-

thological study. He is also indebted to the Central Research Institute of Japan Monopoly Corp. for the infrared spectrophotometric analyses.

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Islanditoxin, a Toxic Metabolite Produced by *Penicillium islandicum* Sopp.

Part II. Acid Hydrolysis of Islanditoxin

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Complete hydrolysis of islanditoxin, $C_{25}H_{33}O_8N_5Cl_2$, with 12 N hydrochloric acid gave three ninhydrin-positive substances, termed fractions A, B and C, and small amounts of a humin-like material. Fractions A and B were identified as serine and α -aminobutyric acid, respectively. To fraction C the molecular formula $C_9H_{11}O_2N$ was assigned and this seemed to be an amino acid containing a benzene ring. Mild hydrolysis of islanditoxin with 4.5 N hydrochloric acid has shown that 1 mole of islanditoxin produces 2 moles of serine.

The isolation, purification, physico-chemical properties and molecular formula of islanditoxin have been described in previous publications.^{1, 2, 3)} The data indicated that islanditoxin has the molecular formula $C_{25}H_{33}O_8N_5Cl_2$ and contains the peptide linkage.

In this report, hydrochloric acid hydrolysis was employed in the hope of obtaining information regarding the components of islanditoxin.

Hydrolysis of islanditoxin with 12 N hydrochloric acid led partially to the formation of a humin-like material. The soluble fraction gave a positive reaction with ninhydrin, while islanditoxin was a ninhydrin-negative substance. Two chromatographies were pursued in the identification of the components of the hydrolysate. First, paper partition chromatography was employed to detect the number of ninhydrin-positive fractions and determine their *RF* values. Second, cellulose

column partition chromatography was studied to isolate each component.

With the partition chromatography, it was indicated that three ninhydrin-positive fractions were finally obtained by the complete hydrolysis with concentrated hydrochloric acid, though about seven ninhydrin-positive spots were found on the paper chromatograms in the course of hydrolysis. They were termed fractions A, B and C in order of their increasing *RF* values.

Fractions A and B isolated in the form of crystals by means of cellulose column chromatography were identified as serine and α -amino butyric acid on the basis of their infrared absorption spectra, *RF* values and melting points. The data of infrared spectra indicated that serine obtained as a fraction of islanditoxin hydrolysate was an optically active form, but optical activity of α -aminobutyric acid could not be established for the lackness of an optically active standard substance. As substitution of the chlorine with the hydroxyl group by hydrolysis

1) S. Marumo and Y. Sumiki; *J. Agr. Chem. Soc. Japan*, **29**, 305 (1955).

2) S. Marumo et. al.; *ibid.* **29**, 913 (1955)

3) S. Marumo; *This Bulletin* **19**, 258 (1955).

happens in some cases, it is not distinct whether serine is an original component of islanditoxin or not. Fraction C was obtained as crystals and its analysis gave an empirical formula $C_9H_{11}O_2N$. This substance seemed to be an amino acid containing a benzene ring on the basis of its ultraviolet and infrared absorption spectra.

To avoid the destruction of serine and the formation of a humin-like material, islanditoxin was hydrolyzed with 4.5 N hydrochloric acid and the amount of serine produced from islanditoxin was determined quantitatively by paper chromatography. The result showed that six carbon atoms of islanditoxin gave two moles of serine and consequently two or more moles of fraction C could not be contained in one molecule of islanditoxin, unrelating to the amount of α -aminobutyric acid contained. Because, the total sum of 2 moles of serine, one mole of α -amino butyric acid and one mole of fraction C as the minimum constitution was $(C_{19}H_{34}O_{10}N_4) - (4H_2O)$ and the balance between this value and molecular formula of islanditoxin $C_{25}H_{33}O_8N_5Cl_2$ was $C_6H_7O_2NCl_2$, it could then be decided that the molecular formula of fraction C is equal to the above empirical formula.

Experimental

Acid Hydrolysis of Islanditoxin

Hydrochloric acid (12 N, 4 ml.) was added to islanditoxin (50 mg.) in a Pyrex test-tube (11 × 200 mm.). The tube was sealed and heated in an oil bath at 105° for 20 hours. The dark colored mixture was evaporated to dryness being added three times with additional quantities of distilled water. The residue was taken up in water (about 5 ml.) and the insoluble humin-like material was separated. The soluble fraction was passed through the anion exchange resin (Amberlite IR 4B) in order to remove hydrogen

chloride and was evaporated to dryness. The colorless residue (43 mg.) was partially applied to the paper chromatography for the qualitative observation of the constitution of islanditoxin and mainly to the cellulose column partition chromatography for isolation of peptide constituents.

Paper Chromatography of Islanditoxin Hydrolysate

Two solvent mixtures were tried on one-dimensional paper partition chromatograms with ascending flow. One of the solvent mixtures was the upper layer of the mixture of butanol, acetic acid and water (4:1:5 by vol.) and the other water saturated phenol. On the paper chromatograms developed by the above solvent mixtures, three ninhydrin-positive spots were obtained and they were termed fractions A, B and C in order of their increasing R_F values. R_F values of A and B were identical with those of serine and α -aminobutyric acid as the guides, respectively. R_F value of fraction C was slightly larger than that of phenylalanine and the development of this spot was weak. On the paper electrochromatograms these three ninhydrin-positive fractions did not move toward the cathode nor anode.

Cellulose Column Partition Chromatography of Islanditoxin Hydrolysate

The chromatographic column (14 × 280 mm.) was placed with cellulose powder by the wetting method, which previously had been successively washed with N hydrochloric acid, water and then solvent mixture of butanol, acetic acid and water (4:1:5 by vol.). The hydrolysate, dissolved in 1.5 ml. of the solvent mixture, was placed on the column and the column was developed in the chamber at 0° with the above solvent mixture at the rate of 2.4 ml./hour. The eluate was collected with every 0.6 ml. by the help of an automatic fraction collector. Each fraction was examined in its color development with the ninhydrin reagent. The tube numbers of the ninhydrin-positive fractions were 48–58 (fraction C), 77–106 (fraction B) and 113–167 (fraction A), corresponding to each spot on the above paper chromatograms, respectively. The eluates containing an individual ninhydrin-positive frac-

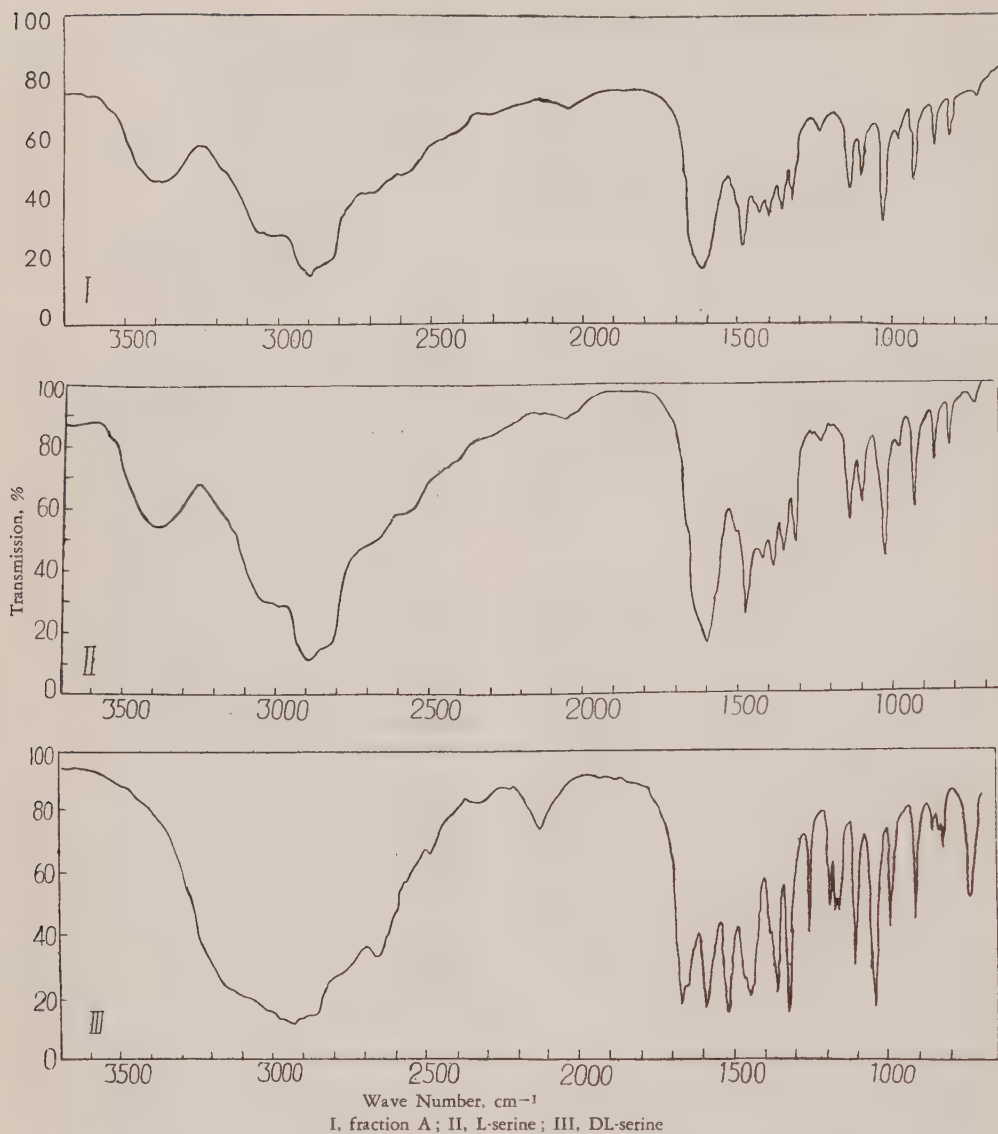


Fig. 1. Infrared Absorption Spectra of Fraction A and Serine (as Nujol mull)

tion were pooled together and evaporated to dryness and all of the three fractions gave almost colorless crystals.

Identification of Fraction A and B

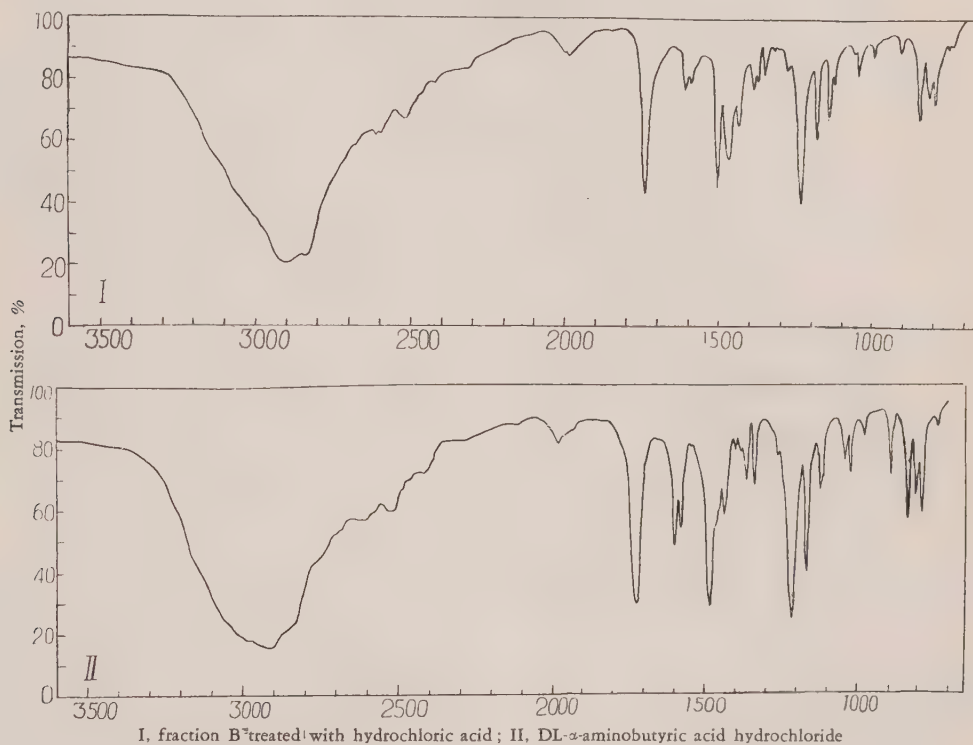
After fraction A and B were washed by absolute ethanol, they were recrystallized from aqueous

ethanol. Fraction A was separated out as short colorless prisms, melting with decomposition at 225–228°, and indicated the positive color reaction of β -hydroxy- α -amino acid with Nessler's reagent. The infrared absorption spectrum of this substance was identical with that of an active form of serine (Fig. 1).

Fraction B treated with hydrochloric acid was obtained as colorless prisms beginning to sublime at 162° and decomposing at $212-214.5^{\circ}$, and was identified with α -amino butyric acid hydrochloride by means of infrared spectrophotometry (Fig. 2).

Fraction C

Fraction C was separated as colorless hexagonal plates from aqueous ethanol and small crystals from a mixture of acetic acid and acetone, sublimating at $220-223^{\circ}$.



I, fraction B treated with hydrochloric acid; II, DL- α -aminobutyric acid hydrochloride
Fig. 2. Infrared Absorption Spectra of Fraction B and α -Aminobutyric Acid (as Nujol mull)

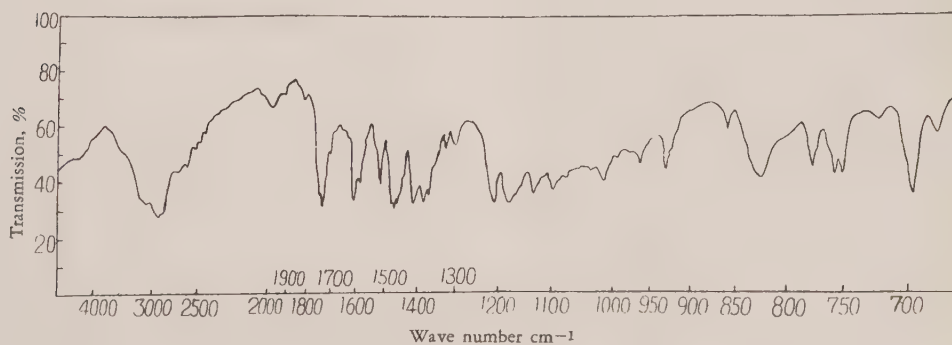


Fig. 3. Infrared Absorption Spectrum of Fraction C treated with Hydrochloric Acid (as Nujol mull)

Table I
Estimation of Serine in Acid Hydrolysates of Islanditoxin.

Islanditoxin hydrolyzed mg.	hours of hydrolysis*	Serine produced found mg.	correct. mg.	molar ratio Serine/Islanditoxin
0.954	6	0.218	0.222	1.33
0.977	8	0.282	0.289	1.69
1.078	12	0.296	0.307	1.62

* concentration of hydrochloric acid: 4.5 N.

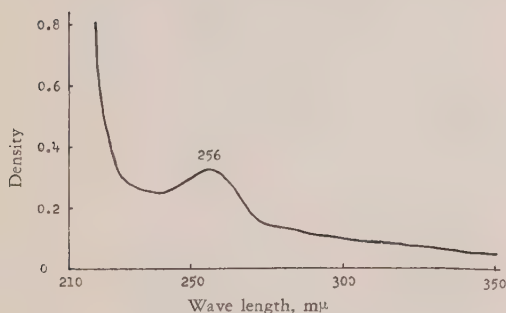


Fig. 4. Ultraviolet Absorption Spectrum of Fraction C in Aqueous Solution.

Anal. Calcd. for $C_9H_{11}O_2N$: C, 65.44; H, 6.71; N, 8.48. Found. C, 65.36; H, 7.44; N, 8.41.

On the basis of the ultraviolet and infrared absorption spectra, this substance appears to be an amino acid containing a benzene ring (Figs. 3 and 4).

Quantitative Estimation of Serine Produced from Islanditoxin

Each of about 1 mg. islanditoxin was hydrolyzed at 105° with 4.5 N hydrochloric acid independently in each of the sealed tubes respectively, and after 6, 8 and 12 hours hydrolysis, the container in each tube was evaporated to dryness three times with additional quantities of distilled water. No humin-like material was formed by hydrolysis. The residue was dissolved in 1 ml. water, and 0.0025 ml. of the aqueous solution was spotted on 2×30 cm. strips of Toyo No. 51 filter paper. After development by the solvent mixture of lutidine, collidine and water (1:1:1 by vol.), the maximum density of each spot was determined by the help of a photoelectric densitometer. The values of the maximum density were corrected in consideration of the destructive effect of the acid hydrolysis on serine. For this cor-

rection, 1 mg. of DL-serine hydrolyzed and treated with the procedure similar to the samples served. By the aid of the maximum density of the spots-concentration of serine curve (Fig. 5), the relationship between the amount of serine found and the time of hydrolysis is shown in Table I.

This fact definitely indicated that 1 mole of islanditoxin produces 2 moles of serine.

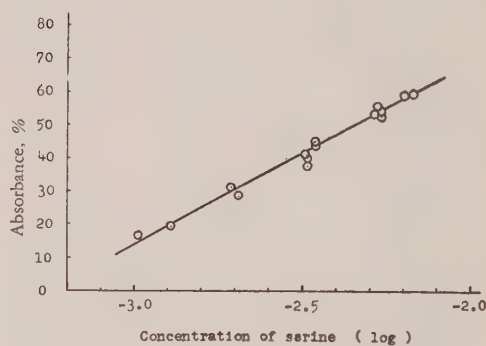


Fig. 5. The Maximum Density of Spots*—Concentration Curve.

* developed by the solvent mixture of lutidine, collidine and water (1:1:1 by vol.) The volumes of samples placed on the paper: 0.0025 mL.

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The authors wish to express their sincere thanks to Prof. Y. Sumiki for his guidance throughout this work, and are also indebted to the Central Research Institute of Japan Monopoly Corp. and Sankyo Co., and Miss S. Hasegawa for the spectrophotometric analyses. The cost of this study was defrayed by the research expenditure of the Food Agency of the Ministry of Agriculture and Forestry, given to Prof. Y. Sumiki, to which authors' thanks are due.

Biochemical Studies on "Bakanae" Fungus

Part XXXIV. Isolation of Gibberellins and Their Properties

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Received October 21, 1955

It has been confirmed that gibberellin A is a mixture of three components, gibberellin A₁, gibberellin A₂ and gibberellic acid (namely gibberellin X), by treating their methyl ester through the chromatography on Al₂O₃ column. Attempts to separate them in free acid were made. The physical and chemical properties of each gibberellin as well as its physiological properties are described.

Gibberellin A and B₁,²⁾ the metabolic products of *Gibberella fujikuroi* which cause abnormal growth of young tissues of higher plants, were isolated and further investigation to elucidate the chemical structure have been published already^{3, 4, 5, 6, 7)}.

Gibberellin A, isolated through the process shown in Fig. 1, was chromatographed on paper using various solvents, butanol-NH₃aq, ethanol-NH₃aq, benzene-acetic acid-water, ethanol-ethyl acetate-ligroin, ethanol-ethyl acetate-benzene, butanol saturated with water, but in every case only one spot was found. Further, as the results of the thirty plates countercurrent distribution method using the solvent system of ethyl acetate and one mole phosphate buffer adjusted to pH 5.3, we found only one peak. From these

facts, we had to arrive at the conclusion, that our gibberellin is a homogeneous compound. However, in contrast to these results, it was found that degradation process³⁾ of gibberellin A to gibberellin C and gibberic acid at the same time, seen in previous reports, could not be explained from the fact that no moiety corresponding to the latter was to be found, and moreover the same results could hardly be obtained by repetition of the experiments. From these contradictory facts, we got to have some doubts on the purity and homogeneity of crystal gibberellin A. At that time, the private communication of April 7, 1954 from Stodola informed us that he had isolated gibberellin X, C₁₉H₂₂O₆ which yields gibberic acid, C₁₈H₂₀O₃ liberating one mole of CO₂ in acid hydrolysis. Fortunately, we succeeded in separating the gibberellin A methyl ester into three different methyl esters on chromatography of Al₂O₃, and it was proved that gibberellin A is a mixture of three components, gibberellin A₁, A₂ and A₃. The empirical formula and physical constants of these three esters are shown in Table I.

It should be emphasized that the an-

1) T. Yabuta, Y. Sumiki: *J. Agr. Chem. Soc. Japan*, **17**, 171 (1938).

2) T. Yabuta, Y. Sumiki, K. Aso, T. Tamura, H. Igara-shi, K. Tamari: *ibid.* **17**, 721, (1941).

3) T. Yabuta, Y. Sumiki, K. Aso, T. Tamura, H. Igara-shi, K. Tamari: *ibid.* **17**, 894 (1941).

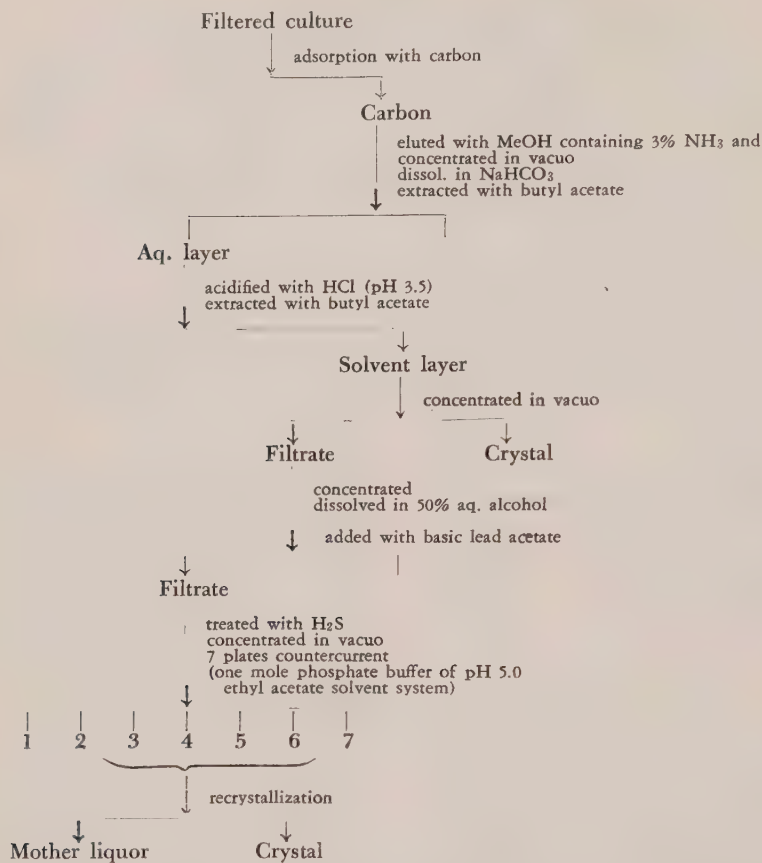
4) T. Yabuta, Y. Sumiki, K. Aso, T. Tamura, H. Igara-shi, K. Tamari: *ibid.* **17**, 975 (1941).

5) T. Yabuta, Y. Sumiki, K. Aso: *ibid.* **25**, 159 (1951).

6) M. Yatazawa, Y. Sumiki: *ibid.* **25**, 503 (1952).

7) Y. Seta, Y. Sumiki: **26**, 508 (1953).

Fig. 1. Process of Separation of Gibberellin A.



alytical data of the methyl ester did not give a satisfactory result in previous reports²⁾. Gibberellin A₃ methyl ester was confirmed to be identical with that of gibberellic acid, which was kindly sent to us by Curtis and Cross^{8,9)} who have isolated this from *Gibberella fujikuroi*, after comparing both infra-red spectra and mixed melting point. Stodola et al¹⁰⁾ also isolated the same substance and named it gibberellin X.

As the publication of gibberellin A₃ has been first reported by Curtis et al

under the name of gibberellic acid, we propose to adapt the name of gibberellic acid.

It was observed that there was a great difference in the recovery of the three components by different strains and composition of culture media. (Table II).

Although we were able to separate each component of gibberellin as its methyl ester, it was necessary to separate the three components in the state of free acid, in order to examine their chemical and physical properties as well as their physiological activities which their esters failed to have shown.

8) Curtis, B. E. Cross: *Chem. and Ind.*, 1954, 1066.

9) B. E. Cross: *J. Chem. Soc.*, 1954, 4670.

10) F. H. Stodola et al: *Arch. Bioch.*, 54, 240 (1955).

Table I
The Empirical Formula and Physical Constants of the Three Esters.

	Formula	mp.	$[\alpha]_D$
Gibberellin A ₁ methyl ester	C ₂₀ H ₂₆ O ₆	230-2°	+35.1°
Gibberellin A ₂ methyl ester			
a) drying in vacuo at room temp.	C ₂₁ A ₃₀ O ₆ ·3/2H ₂ O	190-2°	
b) drying in vacuo at 100° for six hours	C ₂₁ H ₃₀ O ₆ ·1/2H ₂ O	183-5°	
c) drying in vacuo at 100° for twenty hours	C ₂₁ H ₃₀ O ₆	183-5°	+28.1°
Gibberellin A ₃ methyl ester	C ₂₀ H ₂₄ O ₆	202-4°	+67.0°

Table II
The Ratio of Three Components of Crystal Gibb. A from Different Strains.

		A ₁ ester (%)	A ₂ ester (%)	gibberellic acid ester (%)
G-4	crystal	50-60	30-40	<10
	mother liquor	20-30	40-50	20-30
No-4	crystal	70-80	20-30	—
	mother liquor	70-80	20-30	<5

At first, in the purpose to separate gibberellin A₁ and A₂, the crystal gibberellin A from strain No. 4 was used in the following experiments which was fortunately ascertained to be a mixture of gibberellin A₁ and A₂ containing no gibberellic acid, by the chromatography of its methyl ester.

As a direct method of separation, partition chromatography of silica gel was applied. After the preliminary micro test using silica gel column adjusted to pH 4.8, 5.0, 5.2, 5.4, and with the solvent system of CHCl₃-ethyl acetate, CHCl₃-butanol, benzene-butanol, benzene-ethyl acetate, a system of CHCl₃-ethyl acetate and buffer of pH 5.2 was chosen for a large scale. The relation between the fraction number and the recovery curve showed two peaks. But it was proved that every fraction of these two peaks contained gibberellin A₁ and A₂ from the result obtained by the method of ester

chromatography. Adsorption chromatography and ion exchange resin were tried without success.

As indirect methods of separation, attempts were made to prepare their derivatives and separate them taking advantage of the changed physical and chemical properties.

Gibberellin A₁ methyl ester absorbs one mole of hydrogen in catalytic reduction resulting the dihydrogibberellin A₁ methyl ester but gibberellin A₂ methyl ester absorbs no hydrogen. A mixture gibberellin A was hydrogenated catalytically using Adams' catalyser and attempts to separate dihydrogibberellin A₁ and gibberellin A₂ were made by partition chromatography of silica gel resulting in failure in consequence of their similarity of solubility to the various solvents.

In the same manner as the catalytic reduction, gibberellin A₁ methyl ester absorbs bromine but gibberellin A₂ methyl

ester none. The syrup obtained by bromination of gibberellin A mixture at $0^{\circ}\sim 8^{\circ}$ was subjected to chromatography of silica gel. Two kinds of crystal were separated, one of them, monobromo derivative, gives a positive test with the Beilstein reaction and has the formula $C_{19}H_{23}O_6Br$, dp. $215-7^{\circ}$.

Investigation for the purpose of clarifying this peculiar phenomenon, in other words, that we could not obtain the dibromo derivative which should be produced on the addition of one mole of bromine to a double bond, is now under way. The other is the free acid of gibberellin A_2 giving a negative Beilstein test, the formula $C_{20}H_{38}O_6$ being assigned. Its methyl ester is identical with gibberellin A_2 methyl ester in analytical data, mp., and infra-red spectra.

As the separation of methyl ester was

successful, hydrolysis of esters by acid and base to the original acid was tried. When it was treated with $N/10-N/100$ NaOH at room temperature, two kinds of acids were obtained, one of them had the formula $C_{19}H_{24}O_6$, dp. $232-5^{\circ}$, the ester of which is identical with gibberellin A_1 methyl ester in mp. and infra-red spectra. The other is thought to be an epimer of gibberellin A_1 , having the same formula as gibberellin A_1 and should be called pseudogibberellin A_1 . The gibberellin C was obtained by hydrolysis of gibberellin A_1 methyl ester with 20% H_2SO_4 .

It took ten days to hydrolyse gibberellin A_2 methyl ester with $N/10$ NaOH at 30° and the product was assigned to be $C_{18}H_{22}O_6$ which failed to regenerate the original ester in esterification, resulting an ester of mp. $167-9^{\circ}$. Various concentrations of H_2SO_4 were used to hy-

Table III
Abnormal Elongation Growth Test Using Rice Seed (for seven days).

Hights of the stems mm cone. of gibb. %	Gibb. A_1	Gibb. A_2	Gibberellic acid	φ -Gibb. A_1	Gibb. A mixture
0.0016	136	125			121
0.0008	124	118			130
0.0004	109	102	122 (0.0005%)	82 (0.0005%)	118
0.0002	99	93			92
0.0001	83	79			84

control (dist. water) 82.

Table IV
Melting Points and Mixed Melting Points of the Three Components.

	Free acid (dp.)	Me-ester
Gibb. A_1	$232-5^{\circ}$	$232-4^{\circ}$
Gibb. A_2	$235-7^{\circ}$	$190-2^{\circ}$ $183-5^{\circ}$ (anhydrous)
Gibberellic acid	$230-2^{\circ}$	$203-4^{\circ}$
Gibb. A_1 +Gibberellic acid	$232-5^{\circ}$	$211-7^{\circ}$
Gibb. A_2 +Gibberellic acid	$222-4^{\circ}$	$180-4^{\circ}$
Gibb. A_1 +Gibb. A_2	$234-6^{\circ}$	$180-4^{\circ}$

Table V
Analytical Data and Physical Constants of the Three Components.

	Formula	mp.	Specific Rotation	Analytical data (Found)	
A ₁ -free	C ₁₉ H ₂₄ O ₆ C ₂₂ H ₂₆ O ₇	232-5°	+42.3	C: 65.37, H: 7.13	
A ₁ -methyl ester	C ₂₀ H ₂₆ O ₆ C ₂₃ H ₂₈ O ₇	232-4°	+35.1	C: 66.26, H: 6.92	
A ₂ -free	C ₂₀ H ₂₈ O ₆	235-7°	+11.7	C: 66.19, H: 8.01	
A ₂ -methyl ester	C ₂₁ H ₃₀ O ₆ · $\frac{3}{2}$ H ₂ O C ₂₁ H ₃₀ O ₆ · $\frac{1}{2}$ H ₂ O C ₂₁ H ₃₀ O ₆	190-2° 183-5° 183-5°	+28.1	C: 62.11, H: 7.88 C: 65.47, H: 7.77 C: 66.70, H: 7.72	
Gibberellic acid methyl ester	C ₂₀ H ₂₄ O ₆	203-4°	+67.5	C: 66.65, H: 7.34	
	(Required)	Molecular Weight		OCH ₃	pKa
A ₁ -free	C: 65.50, H: 6.94 C: 65.66, H: 6.51	(Found) 370, (titration)	(Required) 348.3 402.4	(Found)	4.9
A ₁ -methyl ester	C: 66.28, H: 7.23 C: 66.33, H: 6.78	382 (OCH ₃)	360 414	8.10	
A ₂ -free	C: 65.91, H: 7.74	360 (titration)	364.4		5.4
A ₂ -methyl ester	C: 62.22, H: 8.15 C: 65.12, H: 8.01 C: 66.64, H: 7.99	372 (OCH ₃) 413 (OCH ₃) 370 (OCH ₃)	405 387 378	8.32 7.51 8.38	
Gibberellic acid methyl ester	C: 66.65, H: 6.71		360.4		

drolyse the ester of gibberellin A₂. Two kinds of crystals were obtained, dp. 255° and 290° as the acidic fraction. The amounts of the former crystal were too small to be analysed. The empirical formula C₁₉H₂₆O₆, was assigned to the latter.

Various attempts to separate free gibberellic acid from a mixture of gibberellin A₁, A₂ and gibberellic acid were made but so far without success.

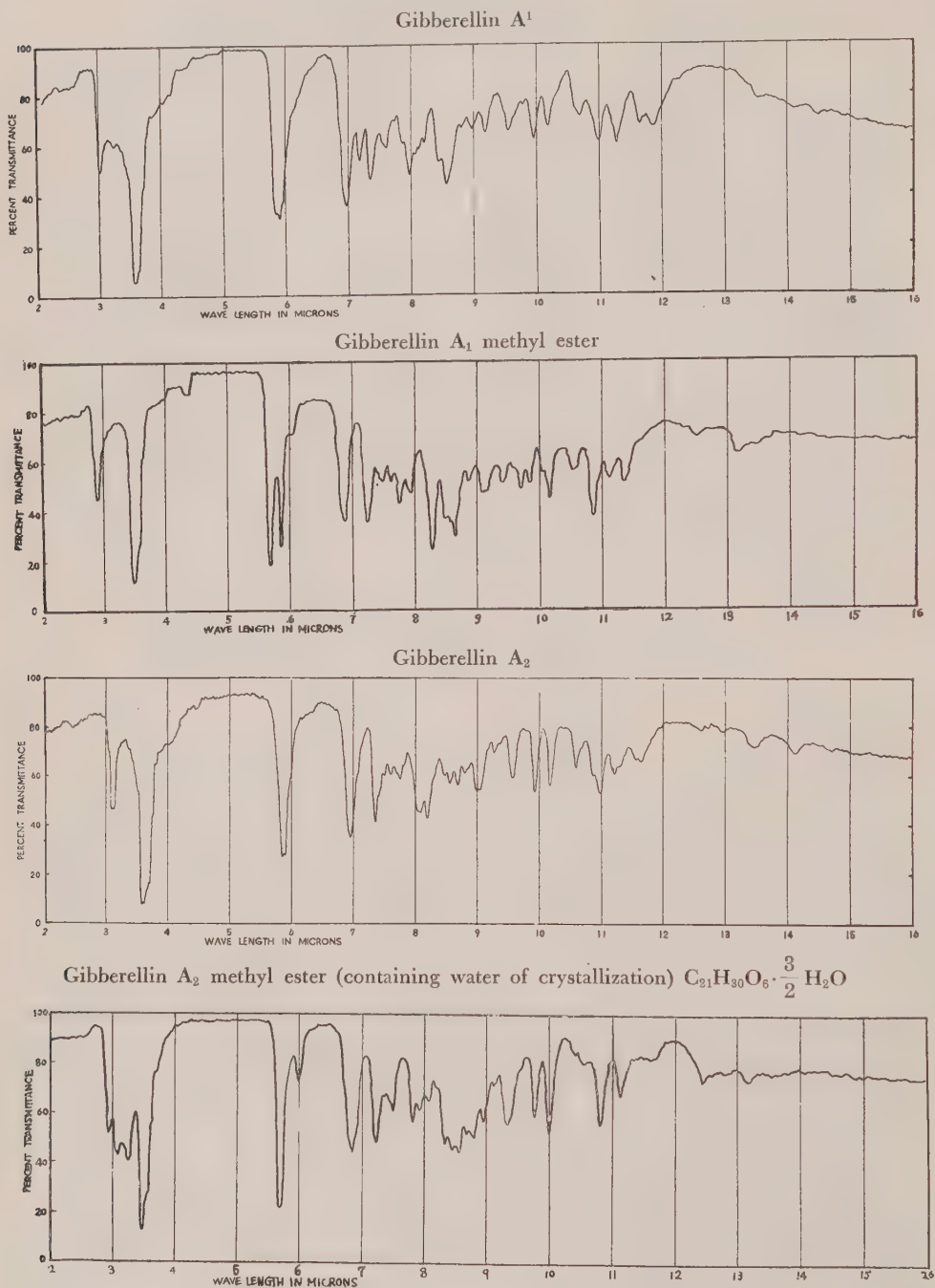
The physiological activities of gibberellin A₁, A₂, pseudogibberellin A₁ isolated in the process described above and gibberellic acid kindly sent to us by Cross were examined. The results are shown in Table III. It was confirmed that the

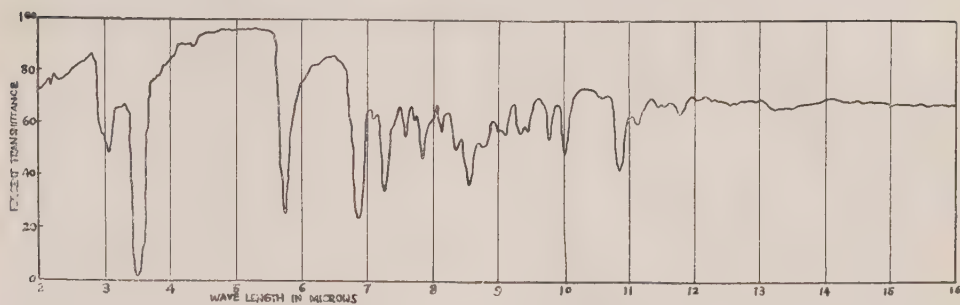
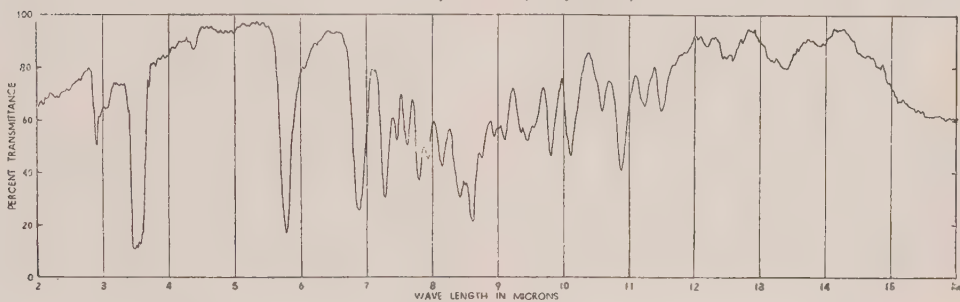
three components of gibberellin A mixture, gibberellin A₁, A₂ and gibberellic acid have strong physiological activities.

The melting point of gibberellin A₁, A₂, gibberellic acid and their esters and also mixed melting point of each two components are shown in Table IV. It is noteworthy that the mixed melting point of each two of the free acids which have a similar melting point do not depress, but although the methyl esters of these acids melt at a somewhat different temperature, their mixed melting point of each two of the three esters show no depression.

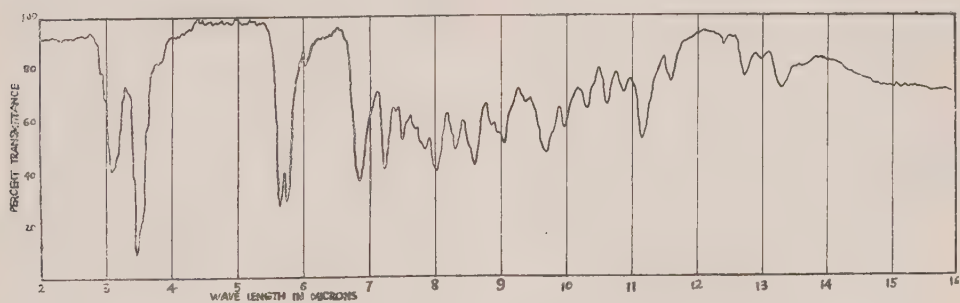
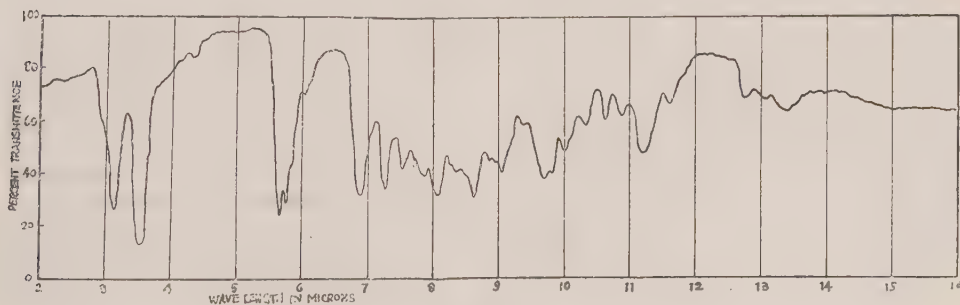
Analytical data and physical constants of the three components are summarized

Fig. 2. Infra-Red Spectra of Free Acids and Their Esters.



Gibberellin A₂ methyl ester $C_{21}H_{30}O_6 \cdot \frac{1}{2} H_2O$ Gibberellin A₂ methyl ester (anhydrous) $C_{21}H_{30}O_6$ 

Gibberellic acid methyl ester

Gibberellin A₃ methyl ester

in Table V.

Ultra violet spectra of gibberellin A₁, A₂, gibberellic acid and their esters showed no maximum in the region of 225 mμ to 300 mμ at the concentration of 5×10^{-3} mol/cc.

Infra-red spectra of free acids and their esters are shown in Fig. 2.

Experimental

1) Paper chromatography of gibberellin A.

On the paper chromatography of gibberellin A using the following solvents at room temperature, only one spot was found.

solvent	indicator	R _F
organic layer of a mixture of (11)		
BuOH and 1.5 N NH ₃ (1:1)	B.C.P.	0.43
a mixture of EtOH and 1.5 N NH ₃ (5:1)	B.C.P.	0.71
organic layer of a mixture of benzene acetic acid and water		
(10:1:5)	B.P.B.	original point
(10:2:5)	„	„
(10:3:5)	„	„
(10:4:5)	„	„
organic layer of a mixture of EtOH, ethyl acetate, ligroin and water (1:1:1:1)	B.P.B.	0.22
organic layer of a mixture of EtOH, ethyl acetate, benzene and water (1:1:1:1)	B.P.B.	0.96
BuOH saturated with water	B.P.B.	0.92
a mixture of benzene and EtOH (6:1)	B.P.B.	0.82 tailing

2) Countercurrent distribution of gibberellin A.

Gibberellin A (50.5 mg) of dp. 240–2° was dissolved in ethyl acetate (10 ml.) and subjected to thirty plates countercurrent distribution with a solvent system of ethyl acetate and one mole phosphate buffer (pH: 5.34). After completion, each buffer layer was extracted with ethyl acetate in the acidic side and each of extract was added to the corresponding upper layer and dried in vacuo. The plate number curve and melting

point of each fraction are shown in Fig. 3.

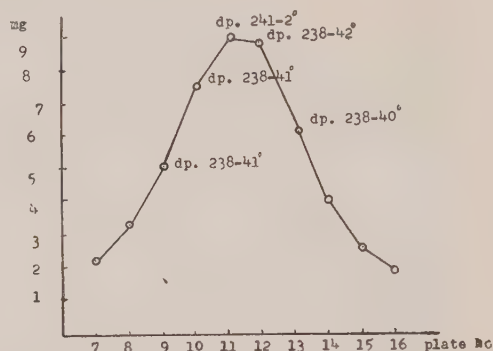


Fig. 3. Relation between Amount of Dried Substance and Plate No. on the Countercurrent Distribution Method.

3) Separation of gibberellin A₁, A₂ and gibberellic acid methyl esters.

Gibberellin A (150mg) from strain G-4 was dissolved in an alcohol ether mixture and treated with an excess of diazomethane in ether solution. On evaporation of the solvent, the residual crystal was dissolved in 20 ml. of ethyl acetate-benzene (1:5), and eluted with the solvent of the same composition, the effluent being collected in every 10ml.

Gibberellin A₁ methyl ester: Fractions No. 10–14 were combined and recrystallized from ethyl acetate-ligroin into prisms, mp. 226–8°, $[\alpha]_D^{25} + 35.1^\circ$ (C=5.04% in methanol).

Gibberellic acid methyl ester: Fractions No. 16–20 were combined (15mg) and after repeated recrystallization, a crystal of mp. 200–2° in fine needles, was obtained, $[\alpha]_D^{25} + 67.0^\circ$ (C=5.38% in methanol).

Gibberellin A₂ methyl ester: At this point, the solvent composition was changed to 1:1 and elution was continued. Fractions No. 23–28 (40mg) were crystallized from the hot ethyl acetate into plates, mp. 190–2°, shrinking at 165–70°, $[\alpha]_D^{15} + 28.1^\circ$ (C=3.33% in methanol).

4) Ratio of the three components for different strains.

The crystal and mother liquor after recrystallization of gibberellin A from the culture media of strains No.-4 and G-4 were esterified with

11) Reid et al: *Biochem. J.* **50**, 60 (1952).

12) Kennedy et al: *Anal. Chem.* **23**, 1033 (1951).

diazomethane and three esters were separated by the chromatography of Al_2O_3 , and their ratio was calculated. The result is shown in Table III.

5) Partition chromatography of gibberellin A.

a) Preliminary test.

As the preliminary test, gibberellin A (15 mg) and silicic acid (5 g) treated with pH. 4.8, 5.0, 5.2, 5.4 buffers (3.5 ml.) and solvent systems of benzene-butanol (95:5), benzene-ethyl acetate (50:50), CHCl_3 -butanol (95:5), CHCl_3 -ethyl acetate (50:50) were used. Every 5 ml. of the effluent was collected and titrated with N/100 alcoholic NaOH. The curve of ml. of effluent and ml. of N/100 NaOH required, showed one peak and one shoulder when the system of CHCl_3 -ethyl acetate and a pH. 5.2 phosphate buffer was used.

b) Large scale experiments.

A sample of 175mg was dissolved in CHCl_3 -ethyl acetate (50:50) 50 ml. The solution was developed on the 30 g silicic acid treated with 25 ml. of pH. 5.2 phosphate buffer. Elution was continued with the same solvent composition. Every 20 ml. of effluent was collected and dried in vacuo. Relation between the amount of dried substance in each fraction and the fraction No. are shown in Fig. 4.

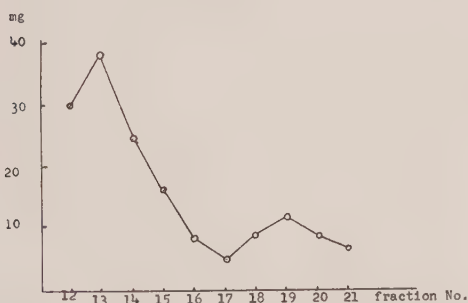


Fig. 4. Relation between Amount of Dried Substance and Fraction No. on the Partition Chromatography.

Fractions No. 12-3, Fraction No. 14 and Fractions No. 18-21 were methylated with diazomethane and the esters thus obtained, were chromatographed on Al_2O_3 to separate the gibberellin A_1 and A_2 methyl esters.

Ratio of A_1 and A_2 obtained:

	$\text{A}_1 : \text{A}_2$	
Fractions. 12-3	7	3
Fract. 14	6	4
Fractions. 18-22	6	4

6) Separation of Gibberellin A_2 by Bromination of a Gibberellin A Mixture.

A gibberellin A mixture was dissolved in 20 ml. of dioxan-ether (1:1). Ether solution of bromine (0.034 g bromine/ml.) was added at $-3^\circ \sim -8^\circ$ for thirty minutes. Additional shaking for two hours was continued at $0^\circ \sim -5^\circ$. The colour of the solution was light brown. About 30 ml. of ether was added and the ether solution was extracted with aq. NaHCO_3 which was acidified and reextracted with ethyl acetate. On evaporation of the solvent, the syrup (320 mg) was obtained. The partition chromatography through silica gel, pretreated with a pH. 5.2 buffer was carried out in preparing the crystal from the syrup using a solvent of butanol-benzene (3:97). A crystal (30 mg) positive to the Beilstein test was obtained at this solvent composition. By-pyramidal obtained on recrystallization from ethyl acetate-ligroin melted at $215-7^\circ$ with decomposition, pKa 4.3.

Anal. Found: C, 53.36; H, 5.36; Br, 18.93.
Calcd. for $\text{C}_{19}\text{H}_{23}\text{O}_6\text{Br}$: C, 53.40; H, 5.39; Br, 18.74. for $\text{C}_{19}\text{H}_{25}\text{O}_6\text{Br}$: C, 53.12; H, 5.83; Br, 18.88.

When the butanol-benzene (10:90) was used as the developing solvent, a crystal negative to the Beilstein test was eluted. On recrystallization from ethyl acetate-ligroin, a crystal of fine needles was obtained, dp. $235-7^\circ$, pKa 5.37, $[\alpha]_D^{15} + 11.7^\circ$ (C=3.16% in methanol).

This is the gibberellin A_2 , the ester of which is identical with gibberellin A_2 methyl ester obtained from the chromatography of ester of gibberellin A mixture, in all respects, the analytical data, mp. and infra-red spectra.

7) Hydrolysis of gibberellin A_1 methyl ester.

a) Acid hydrolysis.

Gibberellin A_1 methyl ester (150 mg) was boiled with 10 ml. of H_2SO_4 (1:5) for one hour. The reaction mixture was extracted with ether continuously and the ether layer was reextracted with aq. NaHCO_3 . The aq. layer was then acidified

and extracted with ether for two days. On evaporating the ether, the residual crystal amounted to 130 mg. By recrystallization, a large amount of plates, dp. 247–9°, and a small amount of needles, dp. 255–7°, were obtained. The substance of dp. 247–9° was found to be gibberellin C from the mixed melting point and analytical data.

Anal. Found: C, 62.26; H, 6.98. Calcd. for $C_{19}H_{26}O_7$: C, 62.28; H, 7.15.

The amount of the crystal of dp. 255–7° was too small to be analysed.

b) Alkali hydrolysis.

i) N/500 NaOH.

Gibberellin A_1 methyl ester (150mg) was shaken with N/500 NaOH (200ml.) for 4 hours at 25°. All crystals were gradually dissolved. The solution was extracted with ethyl acetate at pH 7.5–8.0. On evaporation of the solvent, a crystal (130mg) remained. This was an original substance, mp. 224–5°.

ii) N/100 NaOH.

Gibberellin A_1 methyl ester (150mg) was added to the N/100 NaOH (100ml.). For two days, at a temperature of 30°, all crystals were dissolved, and the solution was extracted with ethyl acetate at pH 8.0 and next pH 2.0. The acidic fraction was a syrup (130mg) which was subjected to the partition chromatography of silica gel using the solvent of butanol-benzene (5:95). In this solvent composition, a small amount of yellow syrup was eluted. Elution was continued with the solvent of butanol-benzene (10:90). At this stage, 35 mg of crystal was eluted and recrystallization from alcohol-ethyl acetate-ligroin gave prisms, dp. 232–5°, $[\alpha]_D^{25} + 42.3^\circ$ (C=3.26 in methanol), pKa 4.9.

The ester of this crystal had dp. 232–4° and its infra-red spectrum was quite similar to that of gibberellin A_1 methyl ester. The mixed melting point of these two crystals indicated no depression. After this crystal was eluted, the solvent was changed to $CHCl_3$ -butanol (80:20). Another crystal (60mg) was eluted and recrystallized from ethyl acetate-alcohol-ligroin into prisms, dp. 225–7°, $[\alpha]_D^{25} + 33.7^\circ$ (C=3.86 in methanol).

Anal. Found: C, 65.62; H, 7.18. Calcd. for $C_{19}H_{24}O_6$: C, 65.50; H, 6.94.

This acid was named pseudogibberellin A_1

which was considered to be an epimer of gibberellin A_1 having the same formula.

Pseudogibberellin A_1 methyl ester was obtained by the reaction of diazomethane and melted at 182–3°.

Anal. Found: C, 66.41; H, 6.97. Calcd. for $C_{20}H_{26}O_6$: C, 66.28; H, 7.23.

On the catalytic reduction of pseudogibberellin A_1 with PtO_2 , one mole of hydrogen was absorbed, resulting dihydropseudogibberellin A_1 , dp. 290°.

Anal. Found: C, 65.12; H, 7.13. Calcd. for $C_{19}H_{26}O_6$: C, 65.12; H, 7.48.

The same result was obtained in the hydrolysis of gibberellin A_1 methyl ester by N/10 and N/50 NaOH.

8) Hydrolysis of gibberellin A_2 methyl ester.

a) Acid Hydrolysis.

Gibberellin A_2 methyl ester (300 mg) and HCl (1:3, 70 ml.) were refluxed for two hours. The reaction mixture was extracted with ethyl acetate. This extract was treated with aq. $NaHCO_3$ and the aq. layer was acidified and reextracted with ethyl acetate. The residue obtained by evaporation of the solvent weighed was 70 mg. This acidic fraction was subjected to the partition chromatography of silica gel treated with a pH 5.2 buffer and solvent of butanol-benzene (5:95), and elution was continued. At this stage, 5 mg of crystal was eluted and recrystallized in needles, dp. 254–5°. The amount of this crystal was too small to be analysed. When the elution was continued with the solvent of butanol-benzene (10:90), 20 mg of crystal was obtained. It was recrystallized from ethyl acetate-ligroin in prisms, dp. 292–4°, mol. wt. 376 by Rast.

Anal. Found: C, 65.26; H, 7.65. Calcd. for $C_{19}H_{26}O_6$: C, 65.14; H, 7.48.

b) Alkali Hydrolysis.

Gibberellin A_2 methyl ester (110mg) was shaken with N/10 NaOH (20 ml.) for ten days at 30°. After the extraction with ether at pH 7.5–8.0 and then 3.0, the acidic fraction (95 mg) recrystallized from alcohol-ligroin in needles, dp. 238–40°.

Anal. Found: C, 64.40; H, 7.33. Calcd. for $C_{18}H_{22}O_6$: C, 64.64; H, 6.63.

Methyl ester of this acid was different from gibberellin A_2 methyl ester in respects of solubility to ethyl acetate and its melting point.

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spectra work necessary for this investigation. This study was supported in parts by the Grant in Aid for Agricultural Research from the Ministry of Education, to which the authors express their thanks.

Biochemical Studies on "Bakanae" Fungus. Part XXXV.

Relation between Gibberellins, A₁, A₂ and Gibberellic Acid.

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Some different results from previous reports on acid degradation and catalytic reduction studies of the three components were obtained. From gibberellin A₁ only gibberellin C and no gibberic acid were obtained. From gibberellic acid (namely gibberellin X and A₂) only gibberic acid and no gibberellin C were obtained. In addition to this, some experimental results of gibberellin A on animal tissues were also, presented.

In part 34 it has been reported that gibberellin A was a mixture of three components, i.e. gibberellin A₁, A₂ and gibberellic acid. For the latter, the name gibberellin X was proposed by Stodola¹⁾ and also named gibberellin A₃ by the authors. But however, as it seems that the name gibberellic acid was first proposed to this substance, in the literature by Curtis et al²⁾, the authors have accepted this name.

In the previous papers^{3,4)} it was reported that acid degradation of gibberellin A₁ gave gibberellin C and gibberic acid at the same time, and the catalytic reduction of gibberellin A dihydrogibberellin A and hexahydrogibberellin A were obtained. As we were able to separate gibberellin A into three components, gibberellin A₁, A₂, and gibberellic acid, a reexamination of these reactions was undertaken whence different results from those previously reported were obtained.

By acid treatment, nothing but gibberellin C could be obtained from gibberellin A₁ while only gibberic acid methyl ester and no gibberellin C were obtained from gibberellic acid methyl ester.

By catalytic reduction, gibberellin A₁ methyl ester absorbed one mole of hydrogen, giving a dihydrogibberellin A₁ methyl ester, while gibberellic acid methyl ester absorbed two moles of hydrogen, giving a tetrahydro-derivative as an oily substance. Hexahydro-derivative was not isolated at all through these experiments.

Owing to the incapability of obtaining the same sample as the one used in previous experiments at present, there is no way to determine whether the former sample was a mixture of several components or not. From the fact that the acid degradation of the former gibberellin A gave gibberellin C, gibberellin B and gibberic acid, it may probably be concluded that the former gibberellin A is at least a mixture of gibberellin A₁ and gibberellic acid. But there some doubt remains that the former gibberellin A may not be a mixture but a substance

1) F. H. Stodola et al: *Arch. Bioch.*, **54**, 240 (1955).

2) Curtis, B. E. Cross: *Chem. and Ind.*, **1954**, 1066.

3) T. Yabuta, Y. Sumiki, K. Aso, T. Tamura, H. Igarashi, K. Tamari: *J. Agr. Chem. Soc. Japan*, **17**, 721 (1941).

4) T. Yabuta, Y. Sumiki, K. Aso, T. Tamura, H. Igarashi, K. Tamari: *ibid.*, **17**, 975, (1941).

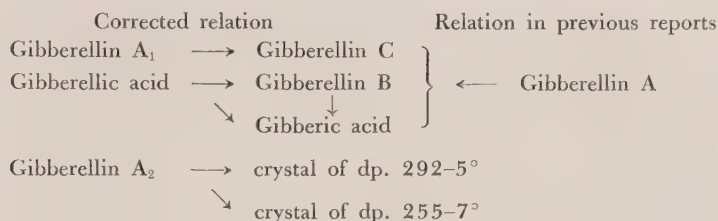


Fig. 1. Relation between each Gibberellin on Acid Degradation.

entirely different from each of the gibberellins, A₁, A₂ and gibberellic acid arising from the previous observation that the melting point of the old gibberellin A, 245°, was found to be higher than that of the other three components, 232-237°, and evolution of carbon dioxide could not be observed in the reaction of the old gibberellin A to gibberic acid, although evolution of one mole of carbon dioxide in the process of gibberellic acid to gibberic acid, was noticed by Cross⁵⁾.

Therefore, a correction of the relation between each gibberellin on acid degradation is necessary and this is summarized in Fig. 1.

Concerning the physiological action of gibberellins upon animal tissues, tested by the tissue culture of a heart of chick embryo, no distinct result was obtained such as that previously reported⁶⁾. In order to ascertain this result, the concentration of gibberellin A was spread to some extent and reexamined. For this purpose, a crystal of gibberellin A, dp. 240-2°, was used to clarify the general physiological action on animal tissues, from a point of view that gibberellin A is a better source than each of the separated gibberellins, A₁, A₂ and A₃. But by means of the injection method to chick embryo applied intravenously not any physiological activity was observed such as is illustrated in the following Tables.

5) B. E. Cross: *J. Chem. Soc.*, **1954**, 4670.

6) T. Yabuta, Y. Sumiki: *J. Agr. Chem. Soc. Japan*, **18**, 207 (1942).

Experimental

Acid degradation.

Gibberellin A₁

Gibberellin A₁ (110 mg) was boiled with 200 ml. of H₂SO₄ aq (1:5) for 30 minutes. On cooling the crystal was precipitated. After extracting the mother liquor with ether, it gave a small amount of crude crystal. Combining the precipitates and recrystallizing them from ethanol-water, yielded prisms, dp. 252-3° (85 mg). The depression was not observed in a mixed melting point with gibberellin C. In the paper chromatography of NH₄-salts of gibberic acid and gibberellin C using NH₄OH-butanol as the solvent, gibberic acid and gibberellin C gave different *R_F* values, which were 0.75 and 0.5 respectively. This method was applied to the crystal and the mother liquor obtained in acid treatment but the spot of gibberic acid could not be noticed.

Gibberellic acid methyl ester

Gibberellic acid methyl ester (100 mg) was refluxed with H₂SO₄ aq (1:5) for one hour. The ether extract at pH 8 gave a crystal (80 mg). Fine needles were obtained by recrystallization, mp. 114°. By the mixed melting point and comparison of the U. V. spectra, it was confirmed that this crystal is identical with gibberic acid methyl ester.

Catalytic reduction.

Gibberellin A₁ methyl ester

Gibberellin A₁ methyl ester was dissolved in 10 ml. of methanol and reduced in hydrogen atmosphere with Adams' catalyser. Hydrogen, 10.5 ml. (ca. one mole), was absorbed for 30 minutes. On evaporation of the solvent, the residue was recrystallized into needles, mp. 234-6°.

Table I

No. of individuals (chick embryos)	Days									
	12	13	14	15	16	17	18	19	20	21
A	1	↓ 0.05c.c.	died
	2	↓	killed
	3	↓	↓ 0.1c.c.	dead-in-shell
	4	↓	↓	hatched
	5	↓	↓	dead
	6	↓	↓ 0.1c.c.	↓	hatched
	7	↓	↓	↓	hatched
	8	↓	↓	↓	killed
	9	↓	↓	↓	killed
	10	↓	↓	killed
	11	↓	↓	died
B	12	↓ 0.05c.c.	↓ 0.1c.c.	↓ 0.1c.c.	killed
	13	↓	↓	↓	hatched
	14	↓	↓	↓	hatched
	15	↓	↓	↓	hatched
	16	↓
	17	↓	↓	↓	killed
	18	↓	↓	↓	killed
	19	↓	↓	died
	20	↓	↓	died
	21	↓ 0.05c.c.	killed
C	22	↓	killed
	23	↓	↓	↓ 0.1c.c.	hatched
	24	↓	↓ 0.1c.c.	↓	killed
	25	↓	↓	↓	hatched
	26	↓	↓	↓	hatched
	27	↓	↓	killed
	28	↓	↓	killed
	29	↓	↓	died
	30	↓	↓	died
	31	↓ 0.05c.c.	↓ 0.1c.c.	killed
D	32	↓	↓	killed
	33	↓	↓	dead-in-shell
	34	↓	↓ 0.1c.c.	↓	killed
	35	↓	↓	↓	hatched
	36	↓	↓	↓	hatched
	37	↓	↓	↓	hatched
	38	↓	↓	↓	died
	39	↓	↓	↓	died

((Division A: gibberellin A 0.1% solution, B: „ 0.001% solution, C: „ 0.00001% solution, D: control (distilled water) ↓: intravenous injection)).

Table II

No. of individuals	Weight of individuals
2	24 g
5	18
A 8	23
9	17
10	24
12	23
B 17	23
18	23
21	21
22	21
C 24	27
27	27
28	24
31	26
D 32	25
34	21

Gibberellic acid methyl ester

Gibberellic acid methyl ester (70 mg) was reduced in the same manner as gibberellin A₁ methyl ester. Hydrogen, 9.8 ml. (ca. 2 moles) was absorbed for 30 minutes. The residual syrup obtained in evaporation of the solvent, could not be crystallized by adsorption chromatography of Al₂O₃ with the use of an ethyl acetate-benzene (1:2) solvent.

Acknowledgement

The animal experiment was undertaken by Prof. S. Yamamoto and K. Fujiwara, Faculty of Agriculture of our University, to whom we are grateful for their kind cooperation. This work was supported in part by a Grant in Aid for Agricultural Research from the Ministry of Education.

Biochemical Studies on Vitamin B₁₂. Part XI.

On Implication of Cobalt-Porphyrin-Derivative in Biosynthesis of Vitamin B₁₂

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In Part X, the authors published studies on vitamin B₁₂-like activity of cobalt-porphyrin-derivative prepared from radish-leaves, and the physiological significance of the compound as an active component in the intermediary metabolism of vitamin B₁₂ was described. Afterward, pure chlorophyll a and b were isolated by column chromatography and the vitamin B₁₂-like activities of Co-porphyrin a and b were repeatedly confirmed.

Recently, effect of Co-porphyrin on the biosynthesis of real vitamin B₁₂ has been tested and the confirmation was also successfully completed by bioautography and paper electrophoresis.

In the present communication, it is discussed as of special importance that cobalt-porphyrin may be the active principle for the formation in vivo of vitamin B₁₂.

In 1954¹⁾, the authors found vitamin B₁₂-like activity of cobalt-porphyrin-derivative obtained from chlorophyll and the physiological role of the compound as one of the active components in the intermediary metabolism of vitamin B₁₂ was reported at Japan Vitamin Society, Tokyo meeting, Sept. 18, 1954. In part X of this series of papers²⁾, after repeated experiments, the alkali- and thermo-stable substance which has the vitamin B₁₂-like activity in plant kingdom was revealed to be a kind of cobalt-porphyrin-complex compound by paper partition chromatography and bioautography, and culture studies on *Euglena gracilis* and *Lactobacillus leichmannii* with cobalt-porphyrin-derivative prepared from radish-leaves were always successful with vitamin B₁₂-free

basal medium when supplemented with synthetic 1,2-diamino-4,5-dimethylbenzene or 5,6-dimethylbenzimidazole.

(1) Further confirmation was obtained by several intensive experiments with cobalt-porphyrin-derivatives prepared from pure chlorophyll a and b. Freshly prepared chlorophyll a and b were converted to cobalt-porphyrin-derivative by treating with CoCl₂ in methanol. The purity of the compound thus obtained was tested by spectrography and paper electrophoresis, and assayed by ordinary *Euglena* method. The results were always positive³⁾.

(2) Moreover, the formation of vitamin B₁₂ with cobalt-porphyrin-derivative prepared in the above experiment was tested by culture of *Euglena gracilis* strains (chlorophyll free) in vitamin B₁₂-free basal medium. Cobalt-porphyrin-derivative supplemented with 5,6-dimethylbenzimidazole

* Faculty of Agriculture, Tokyo University of Education.

1) Y. Sahashi and T. Muto, Japan Vitamin Soc., Tokyo Meeting, 18th, Sept. (1954); *Vitamins* 7, 985 (1954).

Y. Sahashi and T. Muto, Proc. Vitamin B Res. Comm. (Japan) 19th Sept. (1954); *Vitamins* 7, 993 (1954).

2) T. Muto. and Y. Sahashi, This Bulletin, 19 129 (1955).

3) Y. Sahashi and T. Muto, Japan Vitamin Soc., Tokyo Meeting, 17th Sept. (1955); *Vitamins* 9, 361 (1955).

indicated the marked increase of both vitamin B₁₂ in *Euglena* strains and in culture. Vitamin B₁₂ thus prepared was repeatedly studied by bioautography and paper electrophoresis, and the appearance a fair quantity of vitamin B₁₂ was always seen in both the strain and the broth by the nonvitamin B₁₂-producing microbes—*Euglena gracilis* in vitamin B₁₂-free medium⁴⁾.

(3) From these findings the authors have been inclined to propose that cobalt-porphyrin-derivative may be one of the active components in the intermediary metabolism of vitamin B₁₂ in these organisms (Fig. 1).

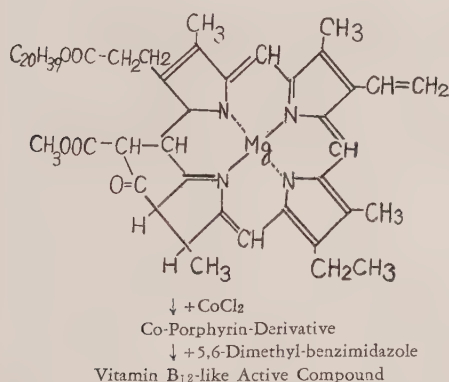


Fig. 1. The Close-Implication of Cobalt-Porphyrin-Derivative for the Biosynthesis of Vitamin B₁₂ (Sahashi and Muto)

(4) Recently, Smith⁵⁾ and Brink⁶⁾ published that they established the chemical structure of vitamin B₁₂, and that the B₁₂ molecule may include a central part

4) Y. Sahashi and T. Muto, Proc. Vitamin B Res. Comm. (Japan) 4th May (1955); *Vitamins* **8**, 518 (1955).

Y. Sahashi and T. Muto, Japan Vitamin Soc., Tokyo Meeting, 6th May (1955); *Vitamins* **8**, 501 (1955).

Y. Sahashi and T. Muto, Proc. Vitamin B Res. Comm. (Japan) 30th July (1955); *Vitamins* **9**, 167 (1955).

5) E. L. Smith, *Biochem. J.* **59**, (No. 4) xxvii (1955); *Chem. Eng. News* **33**, 3487 (1955).

6) Clara Brink, J. Lindsey, J. G. White, J. Pickworth and D. G. Hodgkin, *Nature* **174**, 1169 (1954).

composed of four reduced and heavily substituted pyrrole rings surrounding a single cobalt atom (Fig. 2).

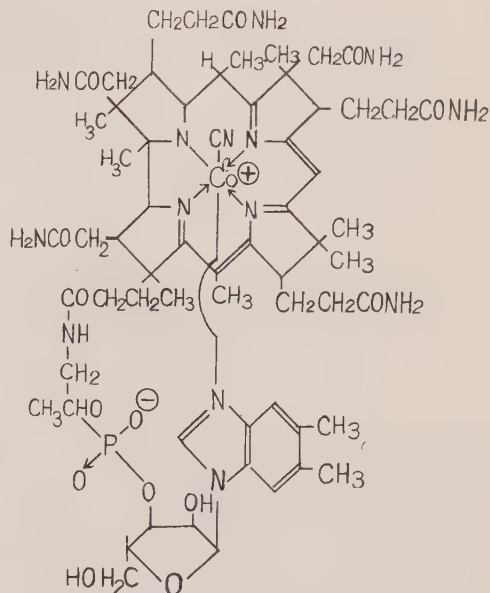


Fig. 2. The B₁₂ Molecule (Smith and Brink)

(5) Of special interest seems to be the close implication of cobalt-porphyrin-derivative in the biosynthesis of vitamin B₁₂ as shown in the following scheme:

Experimental

Preparation of Chlorophyll a and b

Pure chlorophyll a and b were prepared from radish-leaves with the ordinary column chromatography by Gattermann-Wieland⁷⁾ and Winterstein-Stein⁸⁾ methods through a column (2 cm in diameter, 34 cm in length) consisting of 13.5 cm saccharose powder (drying 1 hr at 60–80°C with hot air), 4.5 cm calcium carbonate (drying 1 hr at 150°C) and 8.5 cm active alumina (drying 1 hr at 150°C) with petroleum-ether: benzene (4:1) as mobile phase, as shown in the following table (Table I).

7) L. Gattermann and H. Wieland, *Prax. Org. Chemikers*, **27**, 405 (1940).

8) A. Winterstein and G. Stein, *Z. physiol. Chem.*, **220**, 263 (1934).

Table I
Preparation of Chlorophyll a and b from Radish-leaves.

Air-dried Radish-leaves (300 g)	
extracted with 2.6 l of Petroleumether : benzene : methanol (9 : 1 : 3)	
Filtrate	
washed with 1.6 l of redistilled water	
Dark green petroleumether, benzene Layer	Water, methanol Layer
dried over anhydrous Na_2SO_4 and purified through column chromatography	
Chlorophyll a Chlorophyll b	

Cobalt-Porphyrin-Derivatives from Chlorophyll a and b

Freshly prepared chlorophyll a, 588.1 mg was tested with 500 mg of $\text{CoCl}_2 \cdot 7\text{H}_2\text{O}$ in 25 ml. of methanol at 70°C and after 2 hrs methanol was removed. The bluish-green product was resolved in 75 ml. of ether and subsequently repeated by washing water until no colouration of Co-ion was observed in the aqueous portion for nitroso-R salt. To the ethereal solution thus obtained was added 15% methanolic NaOH solution and adjusted to pH 8. After 72 hrs at 0°C , the required Na salt of black-green Co-porphyrin-derivative(a) separated out in crystalline state, yielding 216.9 mg.

From 322 mg of chlorophyll b, Na salt of Co-porphyrin-derivative(b) was also prepared with the same method mentioned above, yielding 110.7 mg.

Analysis of Cobalt Atom in Co-Porphyrin a and b:
216.9 mg Na salt of Co-porphyrin a gave 19.1mg Co, 5.4% Co
110.7 mg Na salt of Co-porphyrin b gave 10.5 mg Co, 5.7% Co

Absorption Spectra of Co-Porphyrin a and b:

		Reference ⁹⁾
Chlorophyll	a	385, 410, 430, 385, 410, 432,
		507, 667 507, 577, 623, 663,
	b	415, 465, 540, 428, 456, 550
		610, 660 600, 644,

9) R. Willstätter and M. Isler, *Ann. Chem.* **390**, 269 (1912).

Na Salts of { a 410, 420, 510, 600, 650,
Co-Porphyrin { b 425, 510, 600, 640,
rins (10 mg/100 ml. Ethanol) (Benzene)

Paper Electrophoresis: Toyo (No. 50) filter papers, 2×30 cm, were previously treated with phosphate buffer solution, 1/20 M bisodiumphosphate: 1/20 M acidic potassiumphosphate (16:1) (pH 7.8) and dried at room temperature. The samples, 0.1 ml., was dropped on the center line of the papers. Electrophoretic analysis was performed at 3.5 mA, 250 V in direct current (23 – 26°C) in the phosphate buffer solution. After 4 hrs the papers were dried by airing and cut out in five 1cm parts. Then vitamin B_{12} of each part was tested by the ordinary *E. coli* method with B_{12} -free medium supplemented with 6 mg/l of 5,6-dimethylbenzimidazole. The result was as shown in Fig. 3.

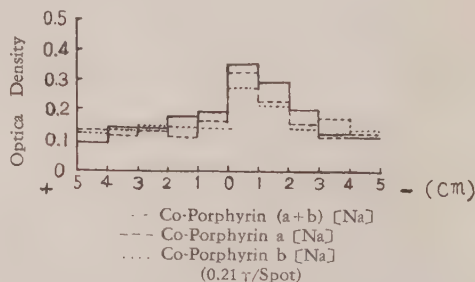


Fig. 3. Paper Electrophoresis of Cobalt-Porphyrin-Derivatives a and b from Chlorophyll.

Table II
Effect of Na Salts of Co-Porphyrin-Derivatives from Chlorophyll a and b
on the Growth of *Euglena*.

Group	Co-Porphyrin a	Co-Porphyrin b	5,6-Dimethyl benzimidazole (DBI)	B ₁₂	CoSO ₄	Optical Density after 144 hrs
a	21mg/l	— mg/l	3 mg/l	— γ /l	— mg/l	0.520
b	—	21	3	—	—	0.565
B ₁₂	—	—	—	0.1	5	0.495
DBI	—	—	3	—	5	0.160
None	—	—	—	—	5	0.140

From the above results, Co-porphyrins a and b appeared in the same position at 0-1 cm from the starting line towards the cathode.

Bioassay of Cobalt-Porphyrin-Derivatives a and b

The effects of Na salt of Co-porphyrin a and b on *Euglena* growth were studied by Co-free basal medium as mentioned above¹⁰⁾ and after culturing 144 hrs at 30°C the growth effect of these compounds on *Euglena* (chlorophyll free) was determined by optical density using a photometer.

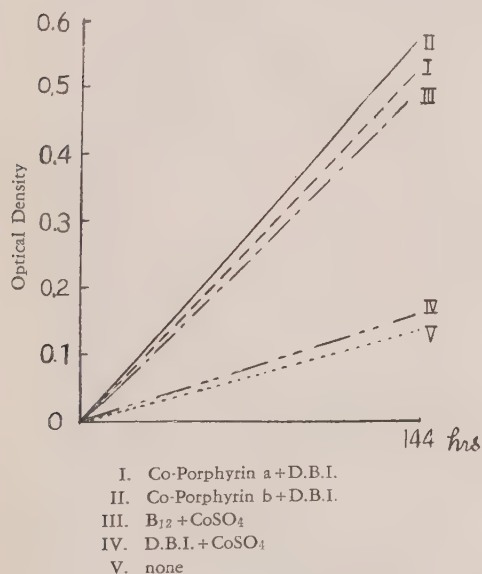


Fig. 4. Effect of Cobalt-Porphyrin-Derivatives a and b from Chlorophyll on the Growth in *Euglena*.

10) T. Muto and Y. Sahashi, This Bulletin 19, 133 (1955).

The vitamin B₁₂-like activity of Co-porphyrins a and b was recognized at the equal response in the case of the supplement with 5,6-dimethylbenzimidazole or 4,5-dimethylphenylenediamine-(1,2) as shown in Table II, Fig. 4.

Effect of Cobalt-Porphyrin-Derivatives for Formation of Vitamin B₁₂ by *Euglena* Strains

Preparation of *Euglena* Strains:

Euglena gracilis was transplanted in the liquid medium from the stock culture agar medium. Ten ml. of its culture solution was added to 90 ml. of B₁₂-free *Euglena* basal medium containing 4.2 mg of Co-porphyrin-derivative and 0.6 mg of 5,6-dimethylbenzimidazole. The medium thus obtained was divided to 10 ml. each in five test tubes, and each was kept at 30°C in the ordinary way. After 72 hours culture, *Euglena* strains were centrifuged (3000 r/min., 10 min.) from the broth, and washed 3 times with sterilized water and transported into three large flasks containing 300 ml. of the B₁₂-free basal medium mentioned above. Again, after 72 hours culture, *Euglena* cells were completely sedimented the broth by centrifuging and washing with sterilized water. To the separated cells was added 10 ml. of redistilled water and adjusted to pH 3.0 with 0.1 N HCl.

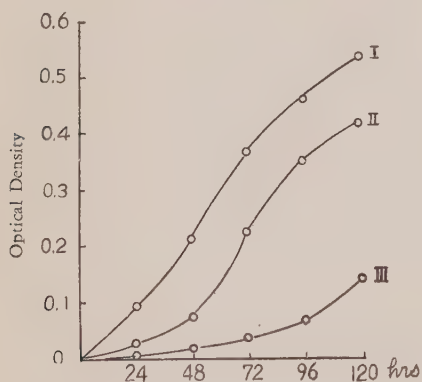
Euglena cells thus obtained was treated with autoclave for 5 min. at 120°C and the residual mass was removed from the solution by centrifuge. The vitamin B₁₂-containing filtrate was adjusted to pH 1.0 with conc. HCl and the greenish precipitates was removed completely by centrifuge, or by extraction with ether. The excess of Co-porphyrin-derivative was again perfectly sedimented free from the vitamin B₁₂-containing preparation.

Table III

Increase of *Euglena* in the Vitamin B₁₂-free Medium supplemented with Co-Porphyrin-Derivative and 5,6-Dimethylbenzimidazole (DBI).

	Exp. treated with 0.01 mγ/ ml. Vitamin B ₁₂	Exp. treated with 42 mg/l Co-porphyrin and 6 mg/l DBI
Amount of <i>Euglena</i> cells in the medium used at the beginning of Exp.	4.69 mg/l	4.69 mg/l
Amount of <i>Euglena</i> cells in the medium obtained at the end of Exp.	427.00 "	264.00 "
Content of V. B ₁₂ in the medium obtained at the end of Exp.	mγ/ml. 0.0555 (medium)	mγ/ml. 0.0014 (cells) 0.0230 " (medium)
Content of V. B ₁₂ in the 1l of the medium obtained at the end of Exp.	mγ/l. 29.12 (medium)	mγ/l 0.087 (cells) 12.07 " (medium)

The broth free from cells was also adjusted to pH 6.0 and evaporated under diminished pressure to 1/10 volume at 40°C after purification mentioned above in the case of the estimation of vitamin B₁₂ in cells. The results were obtained as shown in Table III and Fig. 5.



- I. B₁₂, 0.01 mγ/ml (Broth)
 II. Co-Porphyrin, 42 mg/l + D.B.I., 6 mg/l (Broth)
 III. Co-Porphyrin, 42 mg/l + D.B.I., 6 mg/l (Cells)

Fig. 5. Growth Curve of *Euglena* in the Vitamin B₁₂-free Medium supplemented with Co-Porphyrin-Derivative and 5,6-Dimethylbenzimidazole (DBI).

From the results, the increase of *Euglena* was found in the vitamin B₁₂-free medium by adding Co-porphyrin-derivative and 5,6-dimethylbenzimidazole, but the activity of Co-porphyrin was al-

ways seen rather less in the growth of *Euglena* than in the case of the experiments with vitamin B₁₂. In any case, a fair quantity of vitamin B₁₂ was detected in *Euglena* cells and in the medium even in the vitamin B₁₂-free cultural experiment. The content of vitamin B₁₂ in the medium was always seen more than in the cells.

Further confirmation of vitamin B₁₂ in the broth of *Euglena* was carried out by the ordinary bioautography after treating *Euglena* cells and the medium with enzyme and alkali¹¹⁾.

Enzyme Treatment: The sample 1 ml. was dissolved in 9 ml. of redistilled water, and after adjusting to pH 6.0 was treated with 25 mg of papain and 25 mg of diastase at 37°C with the addition of toluene. After 48 hrs, the solution was heated at 100°C for 30 min., adjusted to pH 4.8, and on cooling 0.1 ml. of 10% metaphosphoric acid was added to remove proteins. To the solution was again added 20 ml. of redistilled water, centrifuged and adjusted to pH 6.5.

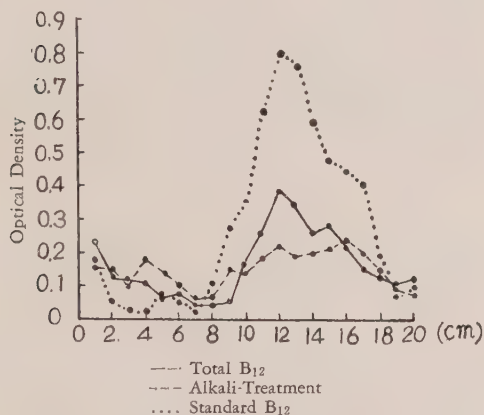
Alkali Treatments: To 1 ml. of the enzyme-treated sample thus obtained was added 8 ml. of 1/5 N NaOH and the mixture was refluxed for 30 min. at 100°C. On cooling pH was adjusted to 6.0~6.5 with 10% HCl, and the solution was evaporated to 1 ml. in vacuo.

Bioautography: Toyo No. 50 filter paper, 2 × 30 cm, was previously treated with 0.66 M acidic potassiumphosphate (pH 4.0) and dried by airing.

11) K. Ueda, "B₁₂ and APF" (Nankodo) 1955, p. 41

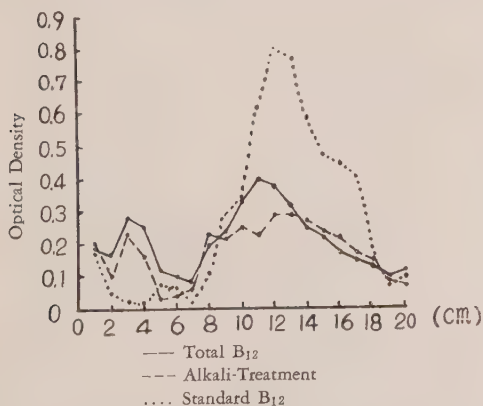
0.1 ml. of the sample purified by enzyme and alkali treatments was spotted on the original line and vitamin B₁₂ was tested by the one-dimensional ascending paper chromatography (*n*-butanol: acetic acid: water=4:1:2 as mobile phase).

The paper was dried by airing and divided into 20 strips of 1 cm each in length. Each strip was taken in the test tube and vitamin B₁₂ was tested respectively by ordinary *Euglena* method. The control experiment was performed with 0.01 ml. per spot of the standard vitamin B₁₂ solution (10 mγ/ml.), Fig. 6, 7.



Mobile Phase: *n*-BuOH: AcOH: H₂O (4:1:2)
Paper-Treatment: 0.66 M KH₂PO₄ (pH 4.0)

Fig. 6. Bioautography of *Euglena* Cells in the Centrifuging Method.



Mobile Phase: *n*-BuOH: AcOH: H₂O (4:1:2)
Paper-Treatment: 0.66 M KH₂PO₄ (pH 4.0)

Fig. 7. Bioautography of the Broth in the Centrifuging Method.

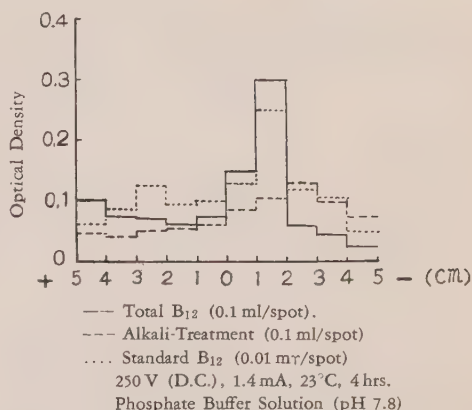


Fig. 8. Paper Electrophoresis of *Euglena* Cells in the Centrifuging Method.

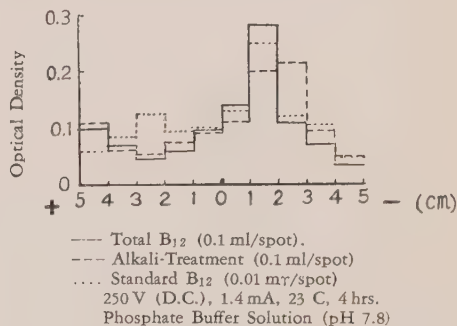


Fig. 9. Paper Electrophoresis of the Broth in the Centrifuging Method.

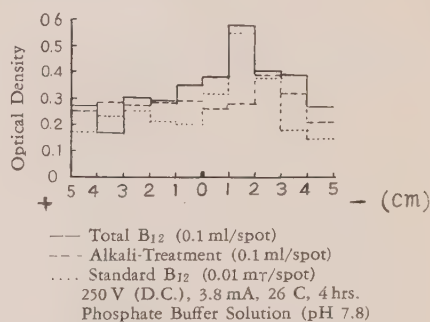


Fig. 10. Paper Electrophoresis of *Euglena* Cells in the Elution Method.

Paper Electrophoresis: Moreover, further intensive studies on the above bioautogram was successively made by paper electrophoresis of the

samples treated with enzyme and alkali as described above. The results are shown in Fig. 8-11.

From the above experiments, it seems to be

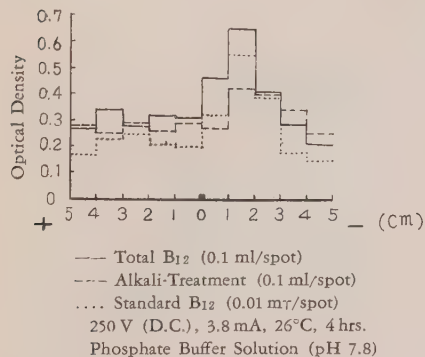


Fig. 11. Paper Electrophoresis of the Broth in the Elution Method.

confirmed that real vitamin B₁₂ may be produced by *Euglena* in the vitamin B₁₂-free medium.

Summary

(1) In the present paper, further confirmation was obtained by repeated intensive experiments with Co-porphyrin-derivatives freshly prepared from pure chlorophyll a and b by the column chromatographic method of Gattermann and Wieland. The effect on *Euglena*

(chlorophyll free) was always revealed to be positive.

(2) Moreover, vitamin B₁₂-mass producing experiments with Co-porphyrin-derivative and 5,6-dimethylbenzimidazole was attempted by *Euglena gracilis* strains in B₁₂-free basal medium, and vitamin B₁₂ was isolated from both cells and broth by paper electrophoresis. The increase of a fair quantity of total vitamin B₁₂ was always seen in the cells and the broth of control strains.

(3) Therefore, the authors are inclined to propose the close implication of cobalt-porphyrin-derivative in the biosynthesis of vitamin B₁₂ by *Euglena*.

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NOTE

Studies on the Detection of Pyruvic Acid.

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Received October 27, 1955

One of the authors M. Yamada found that Rothera's reaction for acetone, ethyl acetoacetate, etc.,¹⁾ was applicable for other ketones in fermentation products and that the reaction could distinguish pyruvic acid from other ketones (permanganate color) by giving a deep blue color.²⁾

H. Caron and D. Raquet applied the same reaction for estimation of lactic acid from which pyruvic acid was produced by oxidation with potassium permanganate.³⁾

Now, we have examined this reaction

more precisely. The procedure of this reaction is as follows.

Place 5 cc. of aqueous solution of sample in a test tube. After saturation with ammonium sulfate, add 1 or 2 drops of 5% sodium nitroprusside solution and 1 or 2 drops of concentrated ammonia solution.

Now we have found that only hexamethylene diamine is suitable for detection in the place of ammonia while other amines such as pyridine, piperidine, aniline, hydroxylamine and methylamine are not. It is possible to replace ammonium sulfate by sodium, potassium or ammonium chloride, but the sharpness of reaction is inferior to that with sulfate.

The following were detected by Rothera's reaction:

1) A. C. H. Rothera; *Journ. Physiol.* **37**, 491-4, 1908, *Chem. Zentr.* **I**, 402, 1909.

2) M. Yamada: *Analysis for Brewers* (in Japanese) p. 143-145, 1936; p.135-137 in the new edition.

3) H. Caron and D. Raquet: *J. Pharm. Chim.* (9) **2**, 333-5, 1942.

Ketones Reagents	Ammonia	Limit of identification	Hexamethylene diamine	Limit of identification
Acetone	violet	50γ	violet	50γ
Pyruvic acid	blue	100	blue	50
Levulinic acid	violet	100	violet	100
Ethyl acetoacetate	orange	100	orange	50
Diacetone alcohol	violet	1000	reddish orange	1000
Methyl ethyl ketone	violet	100	red	1000
Acetophenone	blue	200	blue	1000
Acetoin	violet		orange	
Acetaldehyde	yellow		violet	
Diacetyl	reddish purple		orange	

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